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1. Your reference	SMK/BP5836903
2. Patent application number *(The Patent Office will fill in this part)*	**0106953.3** 20 MAR 2001
3. Full name, address and postcode of the or of each applicant *(underline all surnames)* Patents ADP number *(if you know it)*	The University Court of The University of Aberdeen Regent Walk Aberdeen AB24 3FX UNITED KINGDOM 4267126002
If the applicant is a corporate body, give the country/state of its incorporation	GB
4. Title of the invention	Neurofibrillary Labels
5. Name of your agent *(if you have one)*	MEWBURN ELLIS
Address for service@ in the United Kingdom to which all correspondence should be sent *(including the postcode)*	YORK HOUSE 23 KINGSWAY LONDON WC2B 6HP
Patents ADP number *(if you know it)*	109006

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11. I/We request the grant of a patent on the basis of this application.

Signature | Date 20 March 2001

12. Name and daytime telephone number of person to contact in the United Kingdom — SIMON M KREMER — 020 7240 4405

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Neurofibrillary labels

Field of the invention

The present invention concerns materials, methods and models relating generally to the labelling and detection of neurofibrillary tangles. In addition, it concerns the identification and development of ligands suitable for neuropathological staging and their use in the diagnosis, prognosis or treatment of diseases such as Alzheimer's Disease (AD).

Background to the invention

Neuropathological staging and AD

The neuropathological staging proposed by Braak (Braak, H *et al.* (1991), Acta. Neuropathol. 82, 239-259) provides the best available definition of progression of relatively pure neurofibrillary degeneration of the Alzheimer-type which is diagnostic of AD (Wischik *et al.* (2000), "Neurobiology of Alzheimer's Disease", Eds. Dawbarn *et al.*, The Molecular and Cellular Neurobiology Series, Bios Scientific Publishers, Oxford). This staging is shown schematically in terms of brain region in Fig 2B, and is based on a regular regional hierarchy of neurofibrillary tangle (NFT) distribution. Regions of the brain which appear earlier in the hierarchy have both more tangles and are affected in less severe cases than those later in the list.

Relationship between AD, clinical dementia and neuropathological staging

The provision of an effective pre-mortem assessment of Braak Stage would be of use in the assessment and treatment of AD, for which the differential includes Lewy Body dementia, Parkinson's disease, various forms of fronto-temporal and cortico-basal degeneration, progressive supranuclear palsy and a range of rare neurological syndromes.

The original model proposed by Braak was essentially qualitative in nature, and was not linked to any implications about the threshold for development of clinical dementia and symptoms.

In terms of the appearance of clinical dementia by DSM-IV criteria, this corresponds statistically to the transition between Braak stages 3 and 4

cytoplasm, probably from early tau oligomers which become truncated prior to, and in the course of, PHF assembly (Refs 26,27). They then go on to form classical intracellular NFTs. In this state, PHFs consist of a core of truncated tau and a fuzzy outer coat containing full-length tau (Wischik *et al.* (2000) *loc. cit.*). The assembly process is exponential, consuming the cellular pool of normal functional tau and inducing new tau synthesis to make up the deficit (Ref 29). Eventually functional impairment of the neurone progresses to the point of cell death, leaving behind an extracellular NFT. Cell death is highly correlated with the number of extracellular NFTs (Bondareff, W. *et al.* (1993) Arc. Gen. Psychiatry 50: 350-6). As NFTs are extruded into the extracellular space, there is progressive loss of the fuzzy outer coat of the neurone with corresponding loss of N-terminal tau immunoreactivity, but preservation of tau immunoreactivity associated with the PHF core (Figure 3; Ref 30).

In the process of aggregation, tau protein undergoes a conformational change in the repeat domain associated with a half-repeat phase-shift (Refs 32,33). This creates a proteolytically-stable fragment which is identical to that found in the core of the paired helical filaments (PHFs) which constitute the neurofibrillary tangles characteristic of AD. By analogy with other protein aggregation systems, the process most likely involves an alpha-helix to beta-strand change in conformation (reviewed in Wischik *et al.* (2000) loc. cit.).

Generally speaking therefore, the aggregation of tau can be considered in 3 stages: intracellular oligomers; intracellular filaments; extracellular filaments.

However, to date, no definitive correlation has been established between these stages, which occur at the cellular level, and possibly at different rates and probabilities in different regions in the brain, and the progression of pathology according to the defined hierarchical system of Braak and Braak which, as discussed above, is the best available definition of progression of relatively pure neurofibrillary degeneration.

Invasive methods for assessing AD

Lumbar-puncture CSF measurements enable discrimination between AD and controls, and between AD and other neurological disorders, but lumbar-

(Fig 2c). The DSM-IV criteria (Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, American Psychiatric Association, American Psychiatric Press, Washington DC (1994)) for the definition of dementia equate to an MMSE (Mini-Mental State Examination) cut-off point of about 18, and corresponds to a dementia prevalence of about 5% of the population over-65 years old (over-65's represent about 17% of the total population).

Gertz *et al.* ((1996) Eur. Arch. Psychiatry Clin. Neurosci. 246, 132-6)) studied cases followed from general practice to post-mortem, which were rigorously characterised at the clinical level using CAMDEX (Roth et al, 1988, "The Cambridge Examination for Mental Disorders of the Elderly (CAMDEX)" Cambridge University Press). These were staged post-mortem by the criteria of Braak and, after excluding all cases with any degree of vascular pathology found post-mortem, there remained uncertainty in about one third of cases at the point of transition. That is, about one third of cases with a clinical diagnosis of AD are actually at early Braak stages (stages 1-3), have vascular pathology, or have concomitant Lewy body pathology. Thus there exists a high degree of uncertainty, even in the best practice research setting. The predominant neuropathological substrate that is actually present when a routine clinical diagnosis of AD is made is even more uncertain.

Although it is possible that further refinement in clinical methods with particular reference to more specific neuropsychological indicators (e.g. split attention tasks, delayed matching to sample, etc.) may improve the accuracy of clinical diagnosis, an essential problem is to develop methods for the direct measurement of underlying neuropathology during life, in particular the extent of neurofibrillary degeneration of the Alzheimer-type.

Progression of neurofibrillary degeneration and tau

As described above, the tau-based pathology of AD is a major feature of the phenotype. It is highly correlated with extent of neuronal destruction (reviewed in Wischik *et al.* (2000) *loc cit*).

On a cellular basis, the formation of NFTs from Tau is believed to proceed as follows. In the course of their formation and accumulation, paired helical filaments (PHFs) first assemble as filaments within the

The determination of (ii) above is made based on extracellular aggregated tau. In general terms, for the purposes of the present invention, this may be equated with extracellular tangles (see e.g. Refs 26, 27) and (unless context demands otherwise) the terms may be used interchangeably herein.

It has previously been shown from histological studies that, during the course of aggregation, tau protein acquires binding sites for compounds such as thiazin red and thioflavin-S (Refs 26, 27). The binding site can be shown to exist within the tangle itself, and not in extraneous proteins (Ref 34). Thus both intracellular and extracellular tangles are labelled to some extent by such ligands, as judged histologically.

In general terms, the probability or amount of extracellular binding sites (as opposed to total binding sites, or intracellular sites) may be determined either by using ligands which are too large to gain ready intracellular access, or ligands which can act intracellularly, but at a defined (relatively lower) concentration at which extracellular action is favoured.

Large chelated ligands, such as those susceptible to detection by SPECT, could be expected to at least reach and bind appropriate extracellular targets. Compounds labelled directly for PET could potentially detect both intracellular or extracellular targets, with the latter being favoured at low concentration. Thus the work of the present inventors shows that both of these detection methods have potential in Braak staging, when used with an appropriate tangle-binding ligand. ~~Nevertheless, in the light of the present disclosure, it will be~~ appreciated that in order to conveniently assess Braak stage via NFT numbers it may be important to employ ligands which are not only capable of crossing the blood brain barrier and labelling specified extracellular or intracellular deposits of aggregated tau, but preferably can also retain this property when conjugated to further compounds.

However, for the avoidance of doubt, ligands may be visualised or detected by any suitable means, and the skilled person will appreciate that any suitable detection means as is known in the art could be substituted for these examples.

puncture is more invasive then nuclear medicine-based approaches, and carries a higher risk (Refs. 17 to 21). EEG-neurological diagnosis has also been developed (Refs 22-25), but in this regard there remains a need for cheap instrumentation which can be used at the point of clinician contact.

Neurofibrillary degeneration via brain atrophy - SPECT and PET

Numerous studies have shown that global brain atrophy and specific medial temporal lobe atrophy, particularly of the hippocampus, are closely linked to underlying neurofibrillary degeneration of the Alzheimer-type, and are valuable in the early diagnosis of AD (Refs 1-8).

However, although the diagnosis of AD by monitoring global brain atrophy represents a methodology which can be made to work in a research setting, there are difficulties in defining and measuring atrophy in specific brain regions, and likewise in the measurement of global neocortical atrophy. In any case, a diagnosis based on detectable atrophy may come too late for effective treatment.

There have been advances in diagnostic methodology in recent years, following the identification of diagnostic features in SPECT scans (Refs 9-12; characteristic patterns of perfusion defect detected by HMPAO SPECT), PET scans (Refs 13-15; metabolic defect detected by glucose metabolism profile) and MRI scans (Ref 16; global brain atrophy, specific patterns of lobar atrophy). Of these, the most generally accessible are MRI and SPECT, since PET is for the present time limited to centres which have local specialised cyclotron and radiochemistry capability for the preparation of short half-life injectable radioligands (Aberdeen, London, Cambridge in UK). Notably, the characteristic early stage temporo-parietal perfusion defect detected by HMPAO SPECT in patients with AD corresponds very closely to the pattern of tau pathology which can be detected biochemically (Figure 1). The biochemical changes precede overt neurofibrillary degeneration as seen by the appearance of NFTs (Figure 2; Mukaetova-Ladinska *et al.*, 2000 Am. J. Pathol. Vol. 157, No. 2, 623-636).

However, although MRI and SPECT scans are useful for detecting specific patterns of perfusion defects characteristic of AD, discrimination between different neuropathological stages, or between AD and other types of dementia, is difficult.

For instance, SPECT is useful for detection of a specific pattern of bilateral temporo-parietal perfusion defect that is characteristic of AD (Refs 9 - 11), which can be useful even at very early stage disease. However, SPECT changes discriminate neuropathological stages poorly (Ref 12). Furthermore, discrimination between AD and Lewy Body dementia is difficult. Both have a bilateral temporo-parietal perfusion defect, but only in the latter does an occipitial perfusion defect tend to be present. The same patterns of defect can be demonstrated using PET measurement of glucose metabolism (Refs 13 - 15), but the problem of distinguishing Lewy Body dementia persists in this modality.

Thus, as can be inferred from data in Ref 12, the probability of successful SPECT detection of cases at Braak stages 1&2 is 50%, and at stages 3&4 is 60%. It is only at stages 5&6 that 95% of cases become SPECT-positive. Conversely, cases detected as SPECT-positive could be at stages 1&2 (20%), 3&4 (20%), or 5&6 (60%). SPECT will therefore fail to detect 40 - 50% of the pre-stage 4 target population for early diagnosis and therapeutic intervention.   In a further study (data not shown) it was shown that overall agreement between SPECT diagnosis and clinical diagnosis was of the order of 55%.

Thus, in developing a treatment aimed specifically at preventing neurofibrillary degeneration of the Alzheimer-type, there is a critical need to develop, in parallel, non-invasive means of selecting patients for treatment, and monitoring their response to the treatment, according to a defined and reproducible definition of disease progression.

Disclosure of the invention

Brief summary of the invention

The present inventors have used immunochemical properties (Refs 26, 27, 30) to distinguish intracellular tangles from extracellular tangles. Both the frequency of cases with tangles in these categories (i.e. probability) and their quantity (i.e. counts per mm^2) were determined in a prospective case series and grouped into the regions of the brain known to represent stages in the progression of pathology according to the system of Braak and Braak.

As described in more detail below, these antibody studies demonstrate for the first time that by employing extracellular vs. intracellular specificity, in defined brain regions, deposits of PHF-tau provide a basis for empirical staging of the neurofibrillary degeneration of AD.

Thus in one aspect, the present invention provides a method determining the stage of neurofibrillary degeneration associated with AD in a subject believed to suffer from the disease, which method comprises the steps of:
(i) introducing into the subject a ligand capable of labelling aggregated PHF tau,
(ii) determining the presence and\or amount of ligand bound to extracellular aggregated PHF tau in the medial temporal lobe of the brain of the subject,
(iii) correlating the result of the determination made in (ii) with the extent of neurofibrillary degeneration in the subject.

As described in the introduction, progression of neurofibrillary degeneration is definitive of the neuropathological staging proposed by Braak, which in turn is the best available neuropathological definition of progression of AD. The methods of the present invention can thus be used to provide an actual Braak stage result.

Interestingly, results shown in Gertz *et al.* (1996) *loc cit*, based on immunological detection of NFTs, but which did not distinguish extracellular and intracellular tangles, showed little difference between the numbers detected in demented (generally Braak stage 4-6) and non-demented (generally Braak stage 1-3) subjects in medial temporal lobe structures (see Figure 1 and Table 1, page 134 therein; the relevant structures are labelled Pre alpha ent., CA1, Pri Ento.). Thus the correlation demonstrated by the present invention is particularly surprising.

Some of the aspects of the invention discussed above will now be dealt with in more detail.

Choice of subject

Suitable subjects for the method may be selected on the basis of conventional factors. Thus the initial selection of a patient may involve any one or more of: rigorous evaluation by experienced clinician;

exclusion of non-AD diagnosis as far as possible by supplementary laboratory and other investigations; objective evaluation of level of cognitive function using neuropathologically validated battery.

Ligands

The ligand is capable of labelling aggregated PHF tau, the formation of which is discussed above. It may specifically or preferentially bind such tau (preferentially with respect to competing binding sites present in the relevant region of the brain).

The disclosure that Braak staging can be assessed on the basis described herein has significant implications for the choice and\or development of ligands for use in diagnostic labelling. Immunological methods suffer from the drawback that antibodies do not readily cross the blood-brain barrier in a quantitative manner, and furthermore, the method may be clinically unsuitable since adverse reactions may be triggered by the injection of antibodies into the body for this purpose. It is consequently difficult to discriminate between different stages of tau aggregation on the basis of differential patterns of immunoreactivity in living subjects.

The present inventors have therefore investigated the critical chemical characteristics of compounds which bind to neurofibrillary tangles. They provide herein a minimal chemical structure required for binding which has implications, *inter alia*, in the development and use of compounds as ligands of neurofibrillary tangles and such processes, uses and compounds form further aspects of the invention.

Preferred ligands, including novel ligands, are disclosed in more detail hereinafter, but may include in particular sulphonated-benzothiazole-like compounds (see e.g. Figure 4a) and diaminophenothiazines (see e.g. Figure 8) as well as other mimetic compounds sharing an appropriate minimal chemical structure with either of these. Compositions comprising, or consisting of, combinations of the ligands disclosed herein (preferably distinguishable ligands e.g. in terms of labelling) and\or combinations of ligands with blocking agents (see below) form various aspects of the invention.

Binding to extracellular tau

Enhancement of preferential tau binding

In one embodiment of the invention, steps (i) and\or (ii) of the method are performed in conjunction with (preferably preceded by) the further step of introducing into the subject a second ligand which labels competing (i.e. non-aggregated tau) binding sites present in the relevant region of the brain preferentially to the first ligand.

Thus the methods and other embodiments herein may include a step:

(*ibis*) introducing into the subject a blocking ligand which labels non-aggregated tau binding sites in the brain of the subject preferentially to the ligand capable of labelling aggregated PHF tau.

A competing binding site may be one which is provided by e.g. amyloid plaques, such as may be present in the subject. By introducing such second ligands in to the subject, the relative or effective concentration of first ligand available to bind to aggregated tau may be enhanced. Suitable second ligands (or blocking compounds as they may be described herein) are described below, but they may in particular include benzthiazoles such as are shown in Figure 5, compounds 1B and 2.

Brain regions

The significance of the medial temporal lobe i.e. E2/Trans (Entorhinal cortex layer 2/transitional entorhinal cortex) and E4/HC (Entorhinal cortex layer 4 and hippocampus) regions, and also neocortical structures (FTP), of the brain are demonstrated in Figures 26, 27, 29, ~~and 31.~~

In one embodiment, the method comprises only analysing the data based on extracellular NFTs in the medial temporal lobe.

If a further embodiment, both this region and the neocortical structure data is assessed. In the latter case it may be preferable to assess intracellular PHF deposits.

Thus the methods and other embodiments herein may include a further step:

(iib) additionally determining the presence and\or amount of ligand bound

to intracellular aggregated PHF tau in a neocortical structure of the brain of the subject,

This may be followed by:

(iii) correlating the result of the determination made in (ii) and optionally (iib) with the extent of neurofibrillary degeneration in the subject, and hence the AD state of the subject.

The additional steps may be particularly preferred for assessing or confirming neurofibrillary degeneration in subjects past the point of transition i.e. at Braak stage 4-6 (see e.g. Figure 28, 30, and 32 - Graph labelled FTP).

Determination of neurofibrillary degeneration

The determination may be of the presence of binding in a given area (which can be used to determine a probability) or the amount (density) of binding. Both of these data can be used to generate a Braak stage (see Figures 26-31). The correlation may be done by means of comparison to reference data or some other control. The correlation may be done by means of a look up table or graph e.g. based on data corresponding to the Figures herein (probability, density or amount).

Uses of the method

The determination may be as part of a method of diagnosis or prognosis. It may be used to select a patient for treatment, or to assess the effectiveness of a treatment or a therapeutic e.g. an inhibitor of tau-tau association administered to the subject.

Thus embodiments of the invention include:

A ligand which is capable of labelling extracellular aggregated PHF tau for use in a method of diagnosis or prognosis of AD in a subject believed to suffer from the disease, which method comprises the steps of:
(i) introducing into the subject a ligand capable of labelling aggregated PHF tau,
(ii) determining the presence and\or amount of ligand bound to extracellular aggregated PHF tau in the of the medial temporal lobe of

the brain of the subject,
(iii) correlating the result of the determination made in (ii) with the extent of neurofibrillary degeneration in the subject, and hence the AD state of the subject.

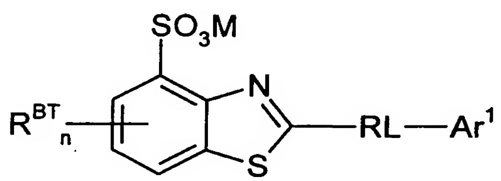
Use of a ligand which is capable of labelling extracellular aggregated PHF tau in a method for preparing a diagnostic or prognostic reagent suitable for use in a method of determining the stage of neurofibrillary degeneration associated with AD in a subject believed to suffer from the disease, which method comprises the steps of:
(i) introducing into the subject said reagent which is capable of labelling aggregated PHF tau,
(ii) determining the presence and\or amount of the reagent bound to extracellular aggregated PHF tau in the medial temporal lobe of the brain of the subject,
(iii) correlating the result of the determination made in (ii) with the extent of neurofibrillary degeneration in the subject.

In a still further aspect, the invention provides a kit for performing the uses and methods described above, the kit comprising one or more ligands or derivatives as provided herein, which are capable of binding to the aggregated molecules. It may include means for increasing the detectability of such compounds e.g. a technetium chelating group, plus optionally means to conjugate this to the ligand, plus optionally technetium. Where the kit comprises a derivative of a compound as disclosed herein, this may be e.g. fluoroscopically detectable, as discussed elsewhere in this description. The kit may also include means for detecting or visualising the ligand, e.g. where the ligand has an incorporated biotin group, the kit preferably includes an antibiotin antibody. Similarly, the kit may include means for detecting the inherent fluorescence of a compound, means for detecting photoactivatable groups, further labelled antibodies, etc.

Various preferred ligands for use in the methods and other embodiments of the present invention will now be discussed in more detail. In each case, those skilled in the art will appreciate that instead of administering ligands directly, they could be administered in a precursor form, for conversion to the active form by an activating agent present in, or administered to, the same subject.

Sulphonated-benzothiazole-like ligands

A suitable ligand for use in this aspect of the present invention is most preferably a ligand compound of the formula I:



wherein:

M is an alkali metal cation;

RL is a rigid linker group;

Ar^1 is an C_{5-20}aryl group;

n is an integer from 0 to 3; and,

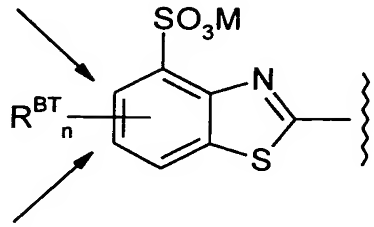
each R^{BT} is a independently benzothiazole substituent.

Each of the rigid linker group, RL, and the aryl group, Ar^1, are substantially planar. In addition, the rigid linker group, RL, and the aryl group, Ar^1, together with the benzothiazole group, form a compound which is substantially planar. By "substantially planar," it is meant that the moiety/compound has a high degree of planarity e.g. less than 5, 4, 3, 2 or 1° twist between the components, as quantified using standard chemical models and assumptions. Preferably the twist will be no greater than that of the compound of Figure 16.

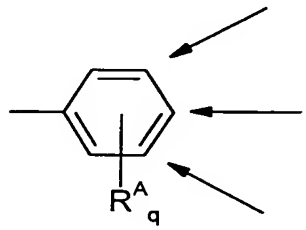
In one embodiment, the compound has a compound length which is from about 14.7 AU to about 15.3 AU.

The present inventors have determined that compounds having the characteristics described above may be particularly suitable for the 'Braak staging' methods of the invention. Such compounds may be known in the art, or may be novel as described in more detail below.

The "compound length" is the distance between the two most distant aromatic ring atoms (denoted "reference atoms"). For example, at the benzothiazole "end" of the molecule, the reference atom will be one of two atoms:



At the aryl "end" of the molecule, when Ar^1 is an aryl group having a phenyl core (see below), the reference atom will be one of three atoms:



Distances used herein may be computed using 'Chemical Database Service', Daresbury, and the Cambridge Structure Database, using 'Chemical structure search and retrieval software'. This data and software are available in the public domain.

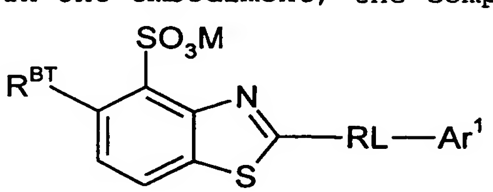
In one embodiment, M is Li, Na, K, or Cs. In one embodiment, M is Na or K.

In one embodiment, n is 0. In one embodiment, n is 1. In one embodiment, n is 2. In one embodiment, n is 3.

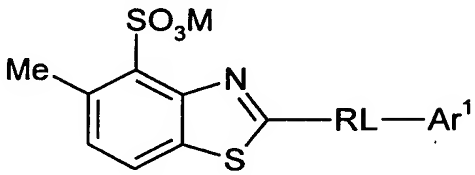
In one embodiment, each R^{BT} is independently selected from: C_{1-4}alkyl, In one embodiment, each R^{BT} is selected from: -Me, -Et, -nPr, and -iPr. In one embodiment, each R^{BT} is -Me.

In one embodiment, n is 1 and R^{BT} is -Me, -Et, -nPr, or -iPr. In one embodiment, n is 1 and R^{BT} is -Me.

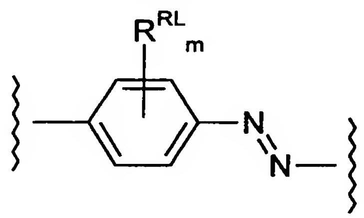
In one embodiment, the compound has the following formula:



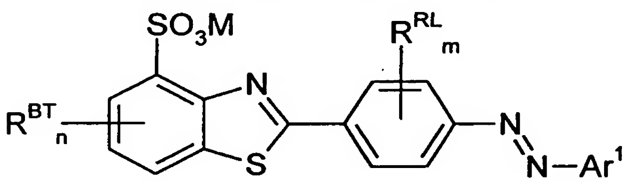
In one embodiment, the compound has the following formula:



In one embodiment, RL is a group of the formula:



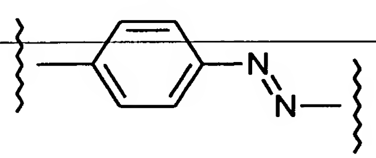
wherein m is an integer from 0 to 4, and each R^{RL} is independently a rigid linker aryl substituent, and the compounds have the formula:



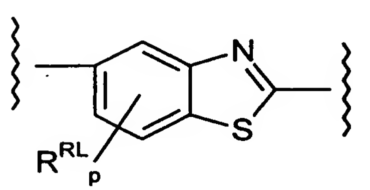
In one embodiment, m is 0. In one embodiment, m is 1. In one embodiment, m is 2. In one embodiment, m is 3. In one embodiment, m is 4.

In one embodiment, each R^{RL} is independently selected from: C_{1-4}alkyl.

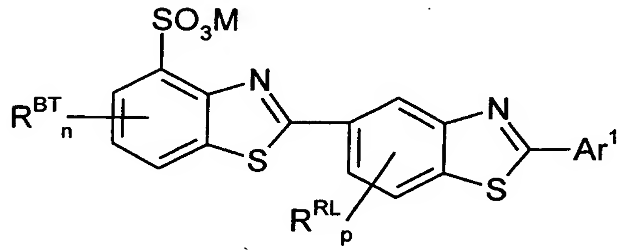
In one embodiment, RL is a group of the formula:



In one embodiment, RL is a group of the formula:



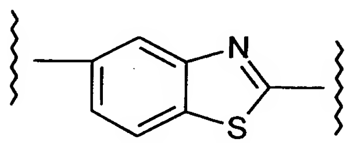
wherein p is an integer from 0 to 3, and each R^{RL} is independently a rigid linker aryl substituent, and the compounds have the formula:



In one embodiment, p is 0. In one embodiment, p is 1. In one embodiment, p is 2. In one embodiment, p is 3.

In one embodiment, each R^{RL} is independently selected from: C_{1-4}alkyl,

In one embodiment, RL is a group of the formula:



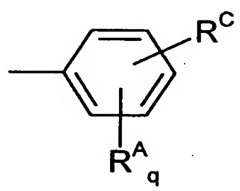
The aryl group, Ar^1, is a C_{5-20}aryl group. The term "C_{5-20}aryl," as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from an aromatic ring atom of a C_{5-20}aromatic compound, said compound having one ring, or two or more rings (e.g., fused), and having from 5 to 20 ring atoms, and wherein at least one of said ring(s) is an aromatic ring. Preferably, each ring has from 5 to 7 ring atoms. "C_{5-20}" denotes ring atoms, whether carbon atoms or heteroatoms.

Examples of C_{5-20}aryl groups which do not have ring heteroatoms (i.e., C_{5-20}carboaryl groups) include, but are not limited to, those derived from benzene (i.e., phenyl) (C_6), naphthalene (C_{10}), anthracene (C_{14}), phenanthrene (C_{14}), naphthacene (C_{18}), and pyrene (C_{16}).

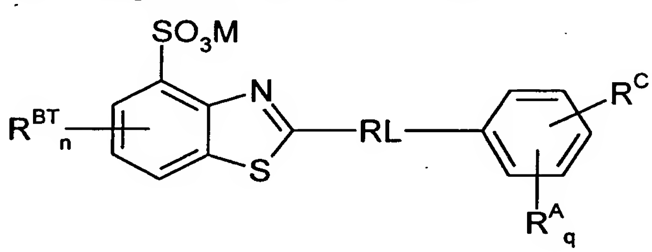
Examples of C_{5-20}heteroaryl groups include, but are not limited to, C_5heteroaryl groups derived from furan (oxole), thiophene (thiole), pyrrole (azole), imidazole (1,3-diazole), pyrazole (1,2-diazole), triazole, oxazole, isoxazole, thiazole, isothiazole, oxadiazole, and oxatriazole; and C_6heteroaryl groups derived from isoxazine, pyridine (azine), pyridazine (1,2-diazine), pyrimidine (1,3-diazine; e.g., cytosine, thymine, uracil), pyrazine (1,4-diazine), triazine, tetrazole, and oxadiazole (furazan).

Examples of C_{5-20}heterocyclic groups (including C_{5-20}heteroaryl groups) which comprise fused rings, include, but are not limited to, C_9heterocyclic groups derived from benzofuran, isobenzofuran, indole, isoindole, purine (e.g., adenine, guanine), benzimidazole; C_{10}heterocyclic groups derived from quinoline, isoquinoline, benzodiazine, pyridopyridine, quinoxaline; C_{13}heterocyclic groups derived from carbazole; C_{14}heterocyclic groups derived from acridine, xanthene, phenoxathiin, phenazine, phenoxazine, phenothiazine.

In one embodiment, Ar^1 is an aryl group having a phenyl core, and has the formula:



wherein q is an integer from 0 to 5; and each R^A is independently an aryl substituent; R^C, is present, is a reactive conjugating substituent; and the compound has the formula:



The reactive conjugating substituent, R^C, if present, is a group which is suitable for conjugation to another molecule.

In one embodiment, the reactive conjugating substituent, R^C, is, or contains, a reactive functional group suitable for conjugation to another molecule by chemical reaction therewith, to form a covalent linkage therebetween. Examples of suitable reactive functional groups include active esters (e.g., succinimidyl esters). In one embodiment, the reactive conjugating substituent, R^C, is, or contains, a moiety suitable for conjugation to another molecule by a strong non-covalent interaction. Examples of such groups include biotin (for binding with molecules bearing avidin or streptavidin). Further R^C substituents are discussed below.

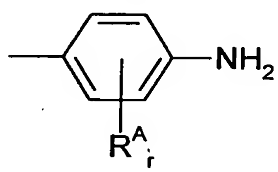
In one embodiment, q is 0. In one embodiment, q is 1. In one embodiment, q is 2. In one embodiment, q is 3.

In one embodiment, q is 4. In one embodiment, q is 5.

In one embodiment, each R^A is independently selected from: $-OH$, $-NH_2$, $-NHR^1$, $-NR^1R^2$, $-SO_3M$, C_{1-4}alkyl, wherein R^1 and R^2 are each C_{1-4}alkyl, and M is an alkali metal cation, as defined above.

In one embodiment, at least one R^A is $-OH$ or $-NH_2$.

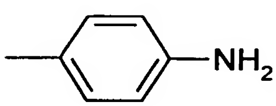
In one embodiment, Ar^1 is an aryl group having an amino-substituted phenyl core, and has the formula:



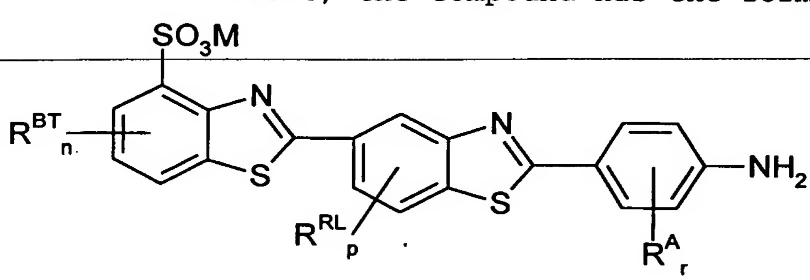
wherein r is an integer from 0 to 4, and each R^A is independently an aryl substituent, as defined above.

In one embodiment, r is 0. In one embodiment, r is 1. In one embodiment, r is 2. In one embodiment, r is 3. In one embodiment, r is 4.

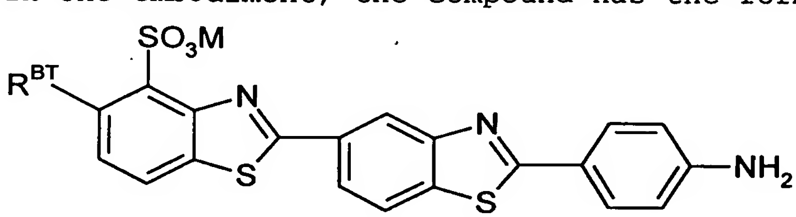
In one embodiment, r is 1 and Ar^1 is a group of the formula:



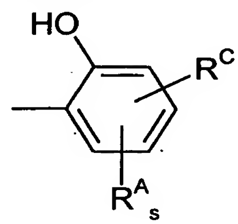
In one embodiment, the compound has the formula:



In one embodiment, the compound has the formula:



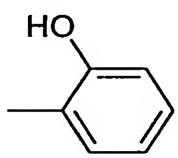
In one embodiment, Ar^1 is an aryl group having a hydroxy-substituted phenyl core, and has the formula:



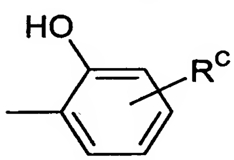
wherein s is an integer from 0 to 4, and each R^A is independently an aryl substituent, as defined above, and R^C, if present, is a reactive conjugating substituent, as defined above.

In one embodiment, s is 0. In one embodiment, s is 1. In one embodiment, s is 2. In one embodiment, s is 3. In one embodiment, s is 4.

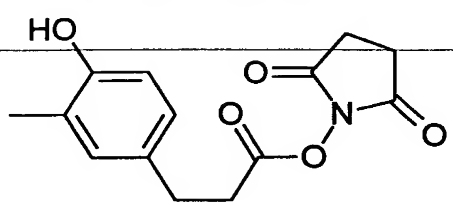
In one embodiment, Ar^1 is a group of the formula:



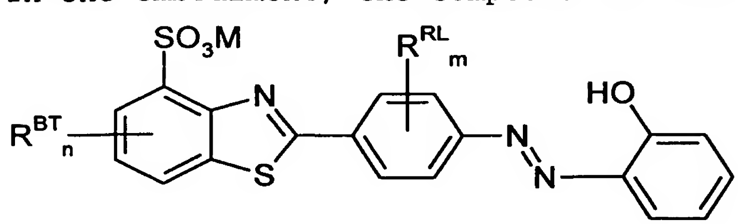
In one embodiment, Ar^1 is a group of the formula:



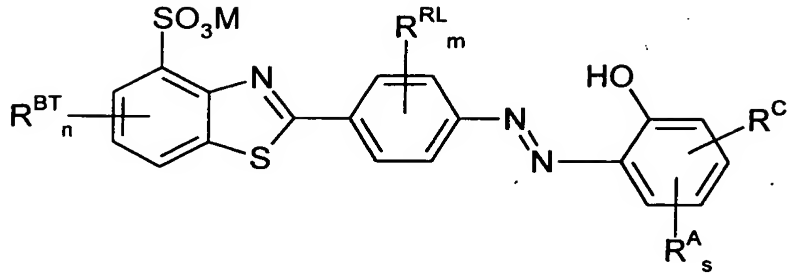
In one embodiment, Ar^1 is a group of the formula:



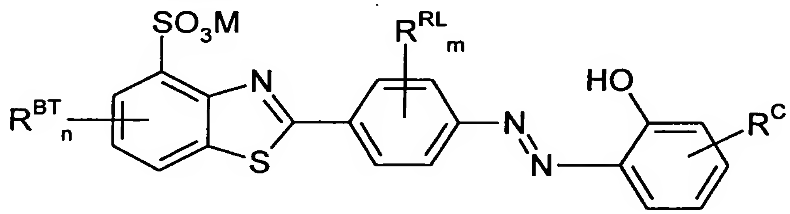
In one embodiment, the compound has the formula:



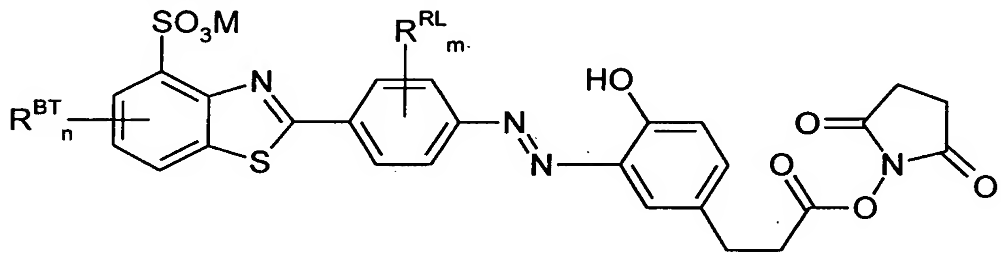
In one embodiment, the compound has the formula:



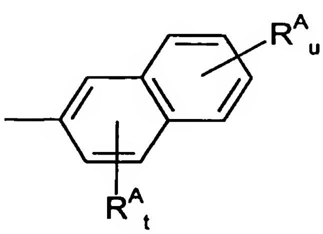
In one embodiment, the compound has the formula:



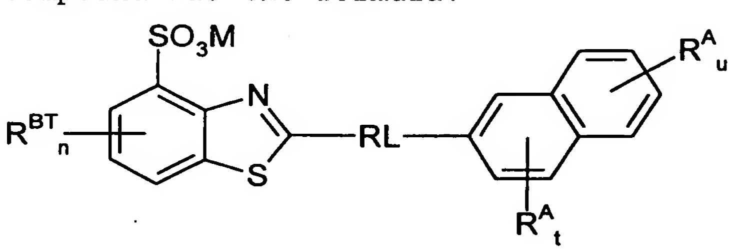
In one embodiment, the compound has the formula:



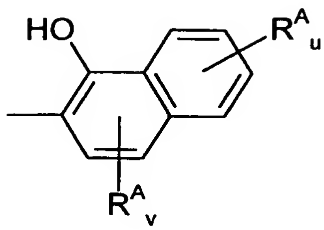
In one embodiment, Ar^1 is an aryl group having a naphthyl core, and has the formula:



wherein t is an integer from 0 to 3, u is an integer from 0 to 4, and each R^A is independently an aryl substituent, as defined above, and the compound has the formula:

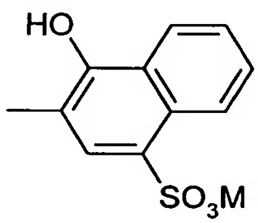


In one embodiment, Ar^1 is an aryl group having a hydroxy-substituted naphthyl core, and has the formula:

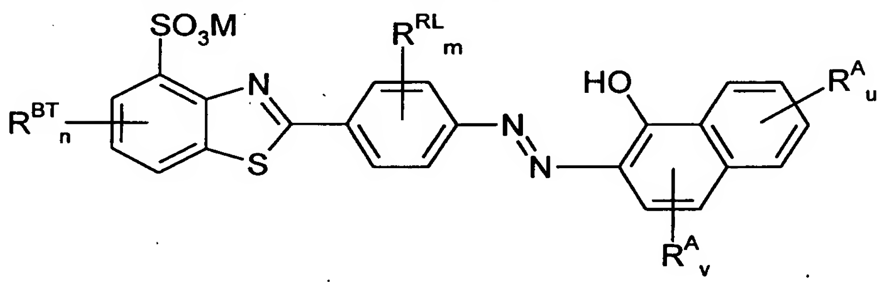


wherein v is an integer from 0 to 2, u is an integer from 0 to 4, and each R^A is independently an aryl substituent.

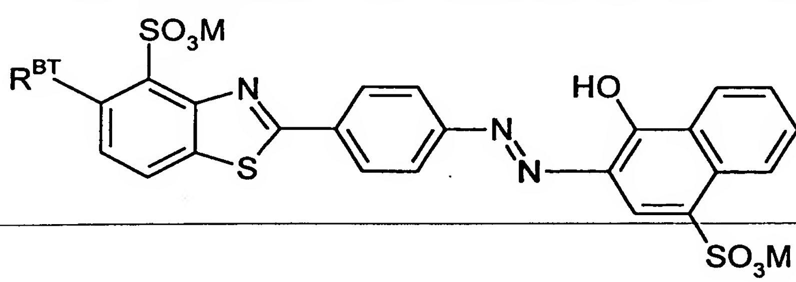
In one embodiment, Ar^1 has the formula:



In one embodiment, the compound has the formula:



In one embodiment, the compound has the formula:



Compounds of the type described above, e.g. of formula I, for use in the diagnostic methods of the present invention, may be prepared my conventional means - see e.g. Reference 31.

All such compounds described herein (or derivatives thereof) having the appropriate formula, size, planarity, and activity may be referred to generally, but not limitatively, hereinafter as 'sulphonated benzothiazole-like compound' or 'SB ligands''). Such compounds will generally be ligands of aggregated tau molecules, e.g. those found in

paired helical filaments or neurofibrillary tangles.

The ligands described herein can suitably be detected by incorporating a positron-emitting carbon into one of the methyl groups of the compound as disclosed herein, and detecting the compound by use of positron emission tomography (PET) as is known in the art. Alternatively, or in addition, a technetium-containing chelate can be incorporated into the compound (e.g. as in the R^c group of the compounds described herein), so that selective detection of extracellular tangles could be achieved. A preferred chelating group is R^C = diethylenetriaminepentaacetic acid.

The ligands may be conjugated, chelated, or otherwise associated, with other chemical groups, dyes, fluorescent markers, antigenic groups, therapeutic moieties, or any other entity which may aid in a prognostic, diagnostic or therapeutic application. For instance, where the ligand is attached to a dye or fluorescent group, the conjugate can be used as a label of aggregated tau or tau-like molecules. It can thus be used to label intracellular or extracellular tangles characteristic of AD.

Alternatively, where the compound is attached to an antigenic group, it can be used e.g. to target therapeutic or diagnostic antibodies to aggregated tau molecules, e.g. extracellular tangles.

Phenothiazines

The present inventors have previously identified another class of compounds, members of which disrupt the structure of PHFs, and reverse the proteolytic stability of the PHF core (WO 96/30766). Such compounds (which may be referred to hereinafter as 'diaminophenothiazines' or 'phenothiazines') include, e.g. tolonium chloride and methylene blue. Examples are shown in Figure 8b.

Phenothiazine compounds described in WO 96/30766, and which may be used in the methods described herein, may be any having a formula shown in Figure 8a, wherein R_1, R_3, R_4, R_6, R_7 and R_9 are independently selected from hydrogen, halogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy.

R_5 is selected from hydrogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy. R_{10} and R_{11} are independently

selected from hydrogen, hydroxy, carboxy, substituted or unsubstituted alkyl, haloalkyl or alkoxy

The term "alkyl" as used in this respect refers to straight or branched chain groups, preferably having one to eight, more preferably one to six, carbon atoms. For example, "alkyl" may refer to methyl, ethyl, n-propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, isopentyl, tert-pentyl, hexyl, isohexyl, and the like. Suitable substituents for the substituted alkyl groups used in the invention include the mercapto, thioether, nitro, amino, aryloxy, halogen, hydroxyl, and carbonyl groups as well as aryl, cycloalkyl and non-aryl heterocyclic groups.

The terms "alkoxy" refers to groups as defined herein above as alkyl groups, as the case may be, which also carry an oxygen atom interposed between them and the substrate residue to which they are attached.

The term "haloalkyl" represents a straight or branched alkyl chain having from one to four carbon atoms with 1, 2 or 3 halogen atoms attached to it. Typical haloalkyl groups include chloromethyl, 2-bromethyl, 1-chloroisopropyl, 3-fluoropropyl, 2,3-dibrombutyl, 3-chloroisobutyl, iodo-t-butyl, trifluoromethyl and the like.

The "halogen" represents fluoro, chloro, bromo or iodo.

Some of these phenothiazines possess one or more asymmetrically substituted carbon atoms and therefore exist in racemic and optically active forms. The invention is intended to encompass the racemic forms of the compounds as well as any of the optically active forms thereof.

Acid addition salts may be formed between basic compounds of formula (I) and inorganic acids, e.g. hydrohalic acids such as hydrochloric acid and hydrobromic acid, sulphuric acid, nitric acid, phosphoric acid etc., or organic acid, e.g. acetic acid, citric acid, maleic acid, fumaric acid, tartaric acid, methanesulphonic acid, p-toluenesulphonic acid etc.

In a particular preferred embodiment the present invention employs a phenothiazine wherein

R_1, R_3, R_4, R_6, R_7 and R_9 are independently selected from -hydrogen, $-CH_3$, $-C_2H_5$, or $-C_3H_7$;

R_{10} and R_{11} are independently selected from hydrogen, $-CH_3$, $-C_2H_5$ or $-C_3H_7$;
R_5 is hydrogen, $-CH_3$, $-C_2H_5$, or $-C_3H_7$ and pharmaceutically acceptable salts thereof.

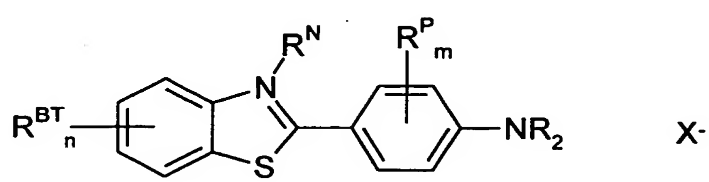
The present inventors now teach herein that such phenothiazine compounds of this sort can bind to the PHFs at a specific site which appears, on the basis of its binding characteristics, to be distinct from that at which the sulphonated benzothiazole-like compounds, described above, can bind. The binding of the phenothiazine compounds to this site is thought to effect the inhibition of tau aggregation.

Phenothiazine compounds may be used in the methods and other embodiments described above, incorporating labels as appropriate. When suitably labeled with a positron-emitting functional group (detectable by PET - see Figures 11, 12, 13) such compounds would serve as ligands for all tau aggregates, and would be capable of crossing the blood-brain-barrier (Ref 36) and entering cells.

In a further embodiment, in the light of the disclosure herein, it will be appreciated that the effect of, and particularly the progress of therapy based on, phenothiazine compounds may be monitorable by use of SB ligands without competitively inhibiting the therapeutic action of the inhibitors. Likewise, therapeutics targeted using SB ligands may be expected to be useful in conjunction with phenothiazine compounds.

Blocking ligands

Preferably these are benzthiazoles of the formula:



wherein:

n is an integer from 0 to 4;
each R^{BT} is independently a benzothiazole substituent;
m is an integer from 0 to 4;
each R^P is independently a phenylene substituent;
each R is independently -H or an amino substituent; and,

either:

R^N and X^- are both absent and the associated (tertiary) nitrogen atom is neutral; or

R^N is a benzothiazolino substituent and the associated (quaternary) nitrogen atom bears a positive charge, and X^- is a counter ion.

Preferred benzthiazoles include thioflavin-S or T. As shown in the Examples below, such compounds (e.g. 1b or 2 in Figure 4) are displaced from NFTs by SB-ligands (e.g. 1). However such compounds do bind preferentially to amyloid.

In one embodiment, n is 0. In one embodiment, n is 1. In one embodiment, n is 2. In one embodiment, n is 3. In one embodiment, n is 4. In one embodiment, n is 0, 1, or 2.

Examples of benzothiazole substituents, R^{BT}, include, but are not limited to, C_{1-4}alkyl groups, $-SO_3H$, and $-SO_3M$, wherein M is a cation. In one embodiment, M is an alkali metal cation. In one embodiment, M is Li, Na, K, or Cs. In one embodiment, M is Na or K. Examples of C_{1-4}alkyl groups include, but are not limited to, -Me, -Et, -nPr, and -iPr.

In one embodiment, each R^{BT} is independently a C_{1-4}alkyl group. In one embodiment, each R^{BT} is selected from: -Me, -Et, -nPr, and -iPr. In one embodiment, each R^{BT} is -Me. In one embodiment, n is 1 and R^{BT} is -Me, -Et, -nPr, or -iPr. In one embodiment, n is 1 and R^{BT} is -Me.

In one embodiment, one of the R^{BT} groups is $-SO_3H$ or $-SO_3M$. In one embodiment, one of the R^{BT} groups is $-SO_3H$ or $-SO_3M$, and another of the R^{BT} groups is a C_{1-4}alkyl group. In one embodiment, n is 2 and one R^{BT} is a C_{1-4}alkyl group and one R^{BT} is $-SO_3H$ or $-SO_3M$. In one embodiment, n is 2 and one R^{BT} is -Me and one R^{BT} is $-SO_3H$ or $-SO_3M$.

In one embodiment, R^N and X^- are both absent and the associated (tertiary) nitrogen atom is neutral.

In one embodiment, R^N is a benzothiazolino substituent and the associated (quaternary) nitrogen atom bears a positive charge, and X^- is a counter ion. Examples of benzothiazolino substituents, R^N, include, but are not limited to, C_{1-4}alkyl groups. In one embodiment, R^N is -Me, -Et, -nPr, or -iPr. In one embodiment, R^N is -Me. Examples of counter ions include,

but are not limited to, Cl^-, Br^-, and I^-. In one embodiment, R^N is -Me and X^- is Cl^-.

In one embodiment, m is 0. In one embodiment, m is 1. In one embodiment, m is 2. In one embodiment, m is 3. In one embodiment, m is 4.

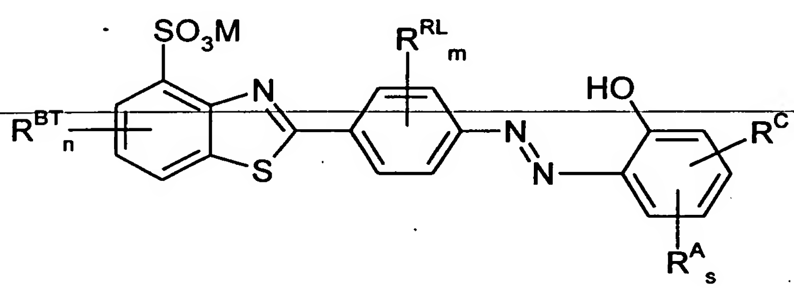
Examples of phenylene substituents, R^P, include, but are not limited to, C_{1-4}alkyl groups.

In one embodiment, each R is -H, and the amino group is $-NH_2$. In one embodiment, one R is -H and one R is an amino substituent. In one embodiment, each R is an amino substituent. Examples of amino substituents include, but are not limited to, C_{1-4}alkyl groups. In one embodiment, the amino group is $-NH_2$, -NHMe, -NHEt, -NH(iPr), -NH(nPr), $-NMe_2$, $-NEt_2$, $N(iPr)_2$, or $-N(nPr)_2$.

Preferred embodiments of blocking ligands are shown in Figure 5 as compounds 1b and 2.

Preferred sulphonated-benzothiazole-like ligands

In one aspect of the present invention, the ligands used to label the aggregated tau, preferable extracellular aggregated tau present in NFTs, are compounds having the formula II:

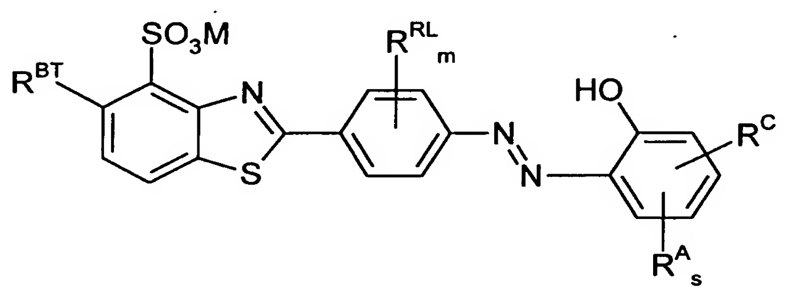


wherein:
M, n, R^{BT}, are as defined and exemplified above with respect to formula I;
each R^{RL} is independently a rigid linker aryl substituent;
s is an integer from 0 to 4;
each R^A is independently an aryl substituent; and,
R^C, if present, is a reactive conjugating substituent.

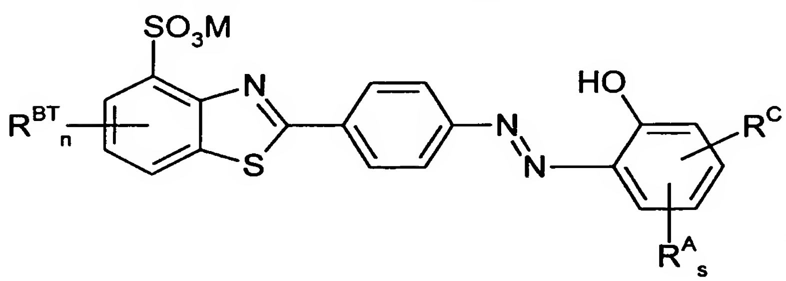
The rigid linker group, RL, and the aryl group, Ar^1, together with the benzothiazole group, form a compound which is substantially planar, that is, has a high degree of planarity.

As shown herein, such compounds may be particularly effective when it is desired to incorporate a bulky R^C group in order to facilitate detection.

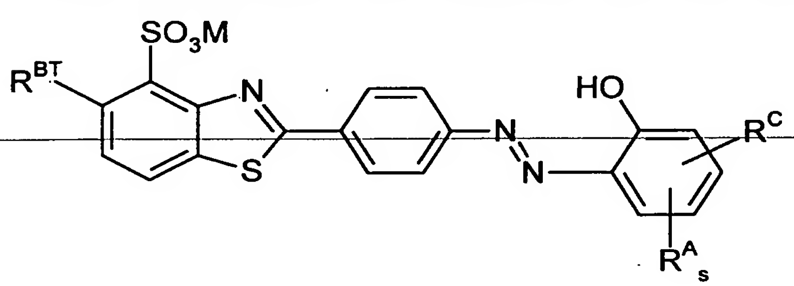
In one embodiment, the compound has the formula:



In one embodiment, the compound has the formula:



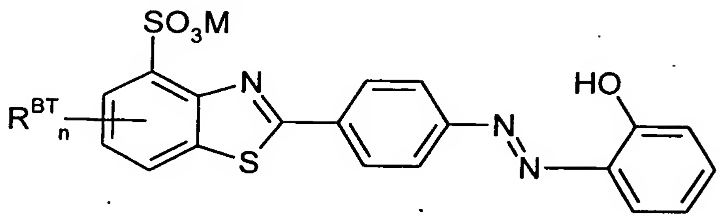
In one embodiment, the compound has the formula:



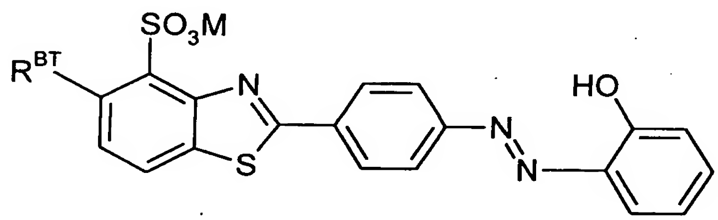
In various embodiments, s may be as discussed above.

In one embodiment, each R^A is independently selected from the substituents given above in relation to formula I.

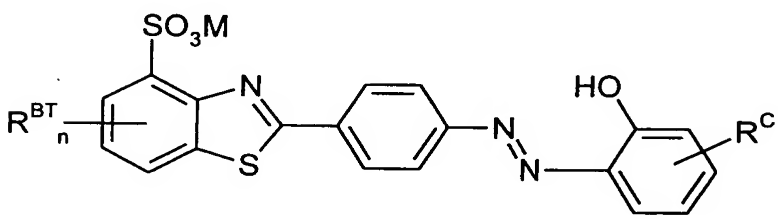
In one embodiment, the compound has the formula:



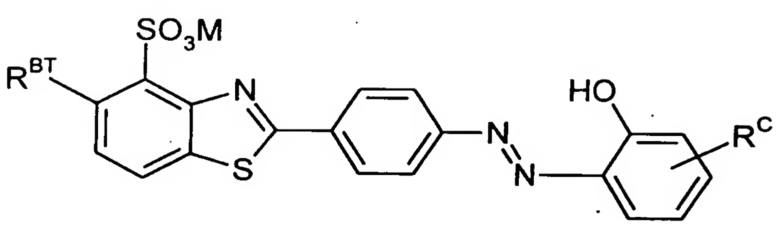
In one embodiment, the compound has the formula:



In one embodiment, the compound has the formula:

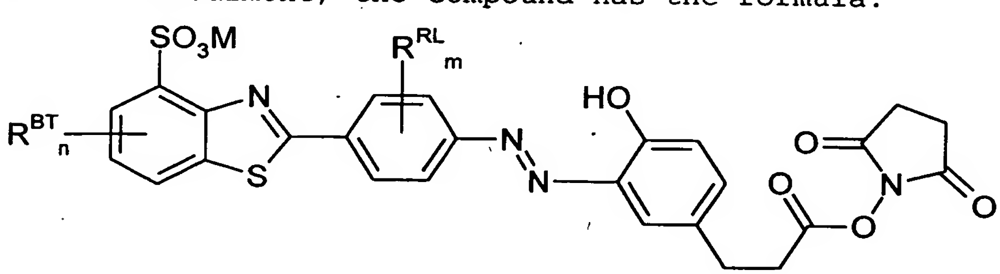


In one embodiment, the compound has the formula:

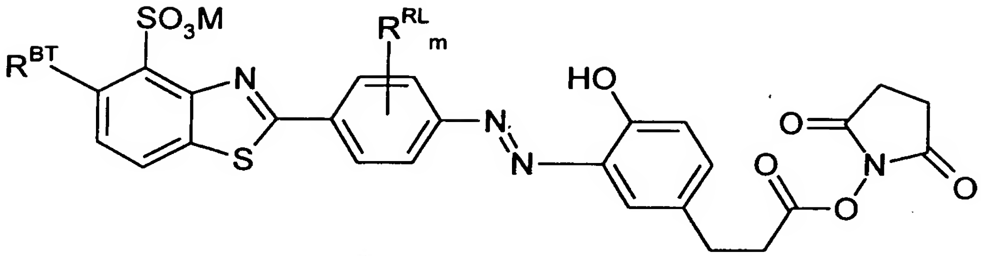


~~Various R^C substituents are discussed elsewhere herein.~~

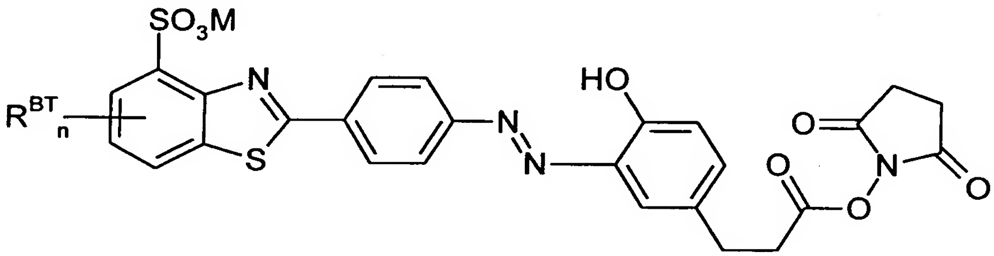
In one embodiment, the compound has the formula:



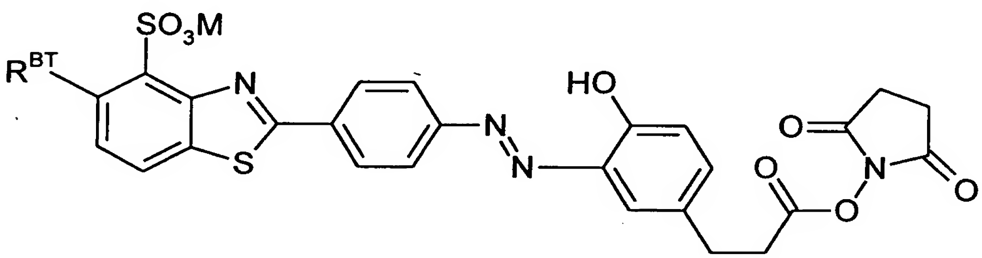
In one embodiment, the compound has the formula:



In one embodiment, the compound has the formula:



In one embodiment, the compound has the formula:



Some of these preferred compounds are shown in Figures 4a-c, and derivatives thereof.

Thus, according to one aspect, the present invention provides a compound represented by the formula shown in Figure 4a, or a derivative thereof e.g. wherein R^c is a conjugating group as described above. As shown in the Examples hereinafter, such derivatives (e.g. compound 4b) retain the appropriate binding activity.

The novel compounds disclosed (e.g. of formula II) herein are useful *inter alia* as synthetic ligands of neurofibrillary tangles, such as those characteristic of AD. The discovery of the minimum critical structure required for binding to these tangles thus provides for the possibility of designing high-affinity ligands which can be used to target the tangles, and can thus be used in the diagnosis, prognosis or treatment of diseases such as AD.

Such compounds will be referred to below as preferred SB ligands.

Mimetics of preferred SB ligands

In general, there are several steps commonly taken in the design of a mimetic from a compound having a given target property (in this case a preferred SB tau-tau aggregation ligand) of which the most important is that the particular parts of the compound that are critical and/or important in determining the target property are determined. The provision by the present inventors of the minimum critical structure required for high affinity binding to aggregated tau molecules has obviated this step.

The minimum critical structure of compound 4a can be modeled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of the ligand, rather than the bonding between atoms) and other techniques can be used in this modeling process.

In a variant of this approach, the three-dimensional structure of the preferred SB ligand and its binding partner are modeled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this in the design of the mimetic. A template molecule is then selected onto which chemical groups which mimic the minimum critical structure can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the required biological activity. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for further testing or optimisation, e.g. *in vivo* or clinical testing. Optimisation may include selecting a mimetic compound as described above, and contacting it with a preparation of aggregated tau molecules (e.g. preaggregated tau in solution, or bound to a solid phase, or isolated from PHFs - see PCT/EP96/01307) and determining the extent to which the test substance(s) binds to the aggregated tau

molecules and\or displaces compound 4a from the molecules.

Compositions

Generally, a preferred SB ligand according to the present invention (e.g. of formula II) may be provided in an isolated and/or purified form, i.e. substantially pure. This may include being in a composition where it represents at least about 90% active ingredient, more preferably at least about 95%, more preferably at least about 98%. Such a composition may, however, include inert carrier materials or other pharmaceutically- and physiologically-acceptable excipients. As noted above and below, a composition according to the present invention may include in addition to a preferred SB ligand as disclosed herein, one or more other molecules of diagnostic, prognostic or therapeutic use.

Methods of labelling aggregated tau

In one aspect, the present invention thus provides a method of labelling aggregated tau or tau-like molecules, comprising contacting the aggregated tau molecules with a preferred SB-ligand compound or derivative thereof as provided herein (e.g. of formula II) and detecting the presence of said compound or derivative. Methods of use may be performed e.g. by analogy to the use of the ligands given in Refs 26-34.

Where used herein, the term "tau protein" refers generally to any protein of the tau protein family. Tau proteins are characterised as being one among a larger number of protein families which co-purify with microtubules during repeated cycles of assembly and disassembly (Shelanski *et al.* (1973) Proc. Natl. Acad. Sci. USA, 70., 765-768), and are known as microtubule-associated-proteins (MAPs). Members of the tau family share the common features of having a characteristic N-terminal segment, sequences of approximately 50 amino acids inserted in the N-terminal segment, which are developmentally regulated in the brain, a characteristic tandem repeat region consisting of 3 or 4 tandem repeats of 31-32 amino acids, and a C-terminal tail.

"Tau like" molecules include, for instance, MAP2, which is the predominant microtubule-associated protein in the somatodendritic compartment (Matus, A., in "*Microtubules*" [Hyams and Lloyd, eds.] pp 155-166, John Wiley and Sons, NY). MAP2 isoforms are almost identical to tau

protein in the tandem repeat region, but differ substantially both in the sequence and extent of the N-terminal domain (Kindler and Garner (1994) Mol. Brain Res. 26, 218-224). Nevertheless, aggregation in the tandem-repeat region is not selective for the tau repeat domain. Thus it will be appreciated that any discussion herein in relation to tau protein or tau-tau aggregation should be taken as relating also to tau-MAP2 aggregation, MAP2-MAP2 aggregation and so on.

The preferred SB ligand may be conjugated, chelated, or otherwise associated with, a further group or entity which has a diagnostic, prognostic or therapeutic purpose or effect, e.g. to a fluorescent group which thus enables visualisation of neurofibrillary tangles to which the ligand binds.

Diagnostic uses

A preferred SB ligand substance according to the present invention, or a composition comprising such a ligand, may be provided for use in a method of diagnosis, prognosis or treatment of the human or animal body by therapy, especially in relation to a condition such as AD.

In a further aspect, the present invention provides a method of diagnosis or prognosis, the method comprising administering to the mammal a diagnostically- or prognostically- effective amount of one or more preferred SB ligands as described herein. This aspect embraces such compounds for use in a method of diagnosis or prognosis. Both *in vitro* and *in vivo* uses are encompassed by this aspect. *In vitro* methods may be performed by (i) obtaining a sample of appropriate tissue from a subject; (ii) contacting the sample with the preferred SB ligand; (iii) detecting the amount and\or localisation of the preferred SB ligand bound to the sample (iv) correlating the result of (v) with the stage or severity of the disease in the subject.

In a further aspect, the present invention provides the use of a preferred SB ligand or derivative as provided herein, in the manufacture of a composition for the diagnosis, prognosis or therapy of a disease as described above.

The disease or condition may be e.g. AD, or an AD-like condition, or any other condition in which aggregated protein molecules are implicated.

Indeed, the assessment of the binding properties of the preferred SB ligand disclosed herein suggests that they may have utility as ligands for diagnosis of any disease in which a protein undergoes an induced conformational change in which α-helix is converted to β-pleated sheet (see Wishchik *et al.*, (2000) *loc it*) - such a conformational change can create a binding site for ligands described herein. Diseases of this type may involve an aggregation cascade may in which induced conformational polymerisation of further protein molecules occurs, leading to the formation of toxic product fragments in aggregates which are substantially resistant to further proteolysis.

Thus it will be appreciated, in the light of the above discussion (and except where context requires otherwise) where the embodiments of the invention are described with respect to tau protein or tau-like proteins (e.g. MAP2) the description should be taken as applying equally to the other proteins discussed above (e.g. β-amyloid, synuclein, prion etc.) or other proteins which may initiate or undergo a similar pathological aggregation by virtue of conformational change in a domain critical for propagation of the aggregation, or which imparts proteolytic stability to the aggregate this formed (article by Wischik *et al.* (in "Neurobiology of AD", 2nd Edition (2000) Eds. Dawbarn, D. and Allen, S.J., The Molecular and Cellular Neurobiology Series, Bios Scientific Publishers, Oxford).

Pharmaceuticals and uses

The ability of the preferred SB ligands disclosed herein to target intracellular and/or extracellular aggregated tau molecules, e.g. in AD brains, provides a useful means for detecting those tau molecules.

However, it may further be used to specifically target an active agent specifically to e.g. neurofibrillary tangles.

Targeting of active ingredients to such aggregated tau may be desirable for a variety of reasons; for example, to bring an agent, which is found to degrade or otherwise render less toxic neurofibrillary tangles in AD brains, into close proximity with the sites at which it would usefully act. Such targeting would be particularly beneficial if the active agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Thus a pharmaceutical composition according to the present invention may comprise as an active ingredient, for example, a derivative of a preferred SB ligand which includes a moiety having a diagnostic, prognostic or therapeutic effect (an 'active moiety') with respect to aggregated tau or tau-like molecules such as those found in neurofibrillary tangles. The part of the derivative which derives from the compound disclosed herein serves to direct the active moiety to the aggregated tau or tau-like molecules, thus providing an effective targeting mechanism for the active moiety of the pharmaceutical. This has potential implications for use in the treatment of conditions such as AD in which aggregated tau molecules are implicated in the pathogenesis of the disease.

The active ingredient is preferably provided in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), by which is meant sufficient to allow detection of aggregated tau molecules, and/or to provide a therapeutic or prophylactic effect or benefit to the individual as appropriate. The actual amount administered, and rate and time-course of administration, will depend on the concentration and/or locality of the aggregated tau molecules in the cells, and/or the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

Pharmaceutical compositions may comprise, in addition to one of the above SB-ligand derivatives, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser, or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the binding activity of the substance to aggregated tau, or the efficacy of any bioactive group linked to or otherwise associated with the substance. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the ligand will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

A composition as described above may be administered alone or in combination with other treatments, either simultaneously or sequentially, depending on the condition to be treated.

These, and other, aspects of the present invention will become more apparent on reading the ensuing non-limiting Examples, in which embodiments of the invention will be described by way of example only. Reference is made to the accompanying figures, in which:

<u>Brief description of the Figures</u>

Figure 1 shows the regional distribution of PHF-tau, measured using antibodies mAb423 (A) or mAb7.51 after formic acid treatment of the PHF fraction (B), for 18 cases of AD. Taken from Mukaetova-Ladinska *et al.*, (1993), Am. J. Pathol. 143, 565-578.

Figure 2 (a) shows the aggregation of tau molecules and the appearance of neurofibrillary tangles during the pathological stages of AD. Taken from (Mukaetova-Ladinska, E.B. *et al.* (2000) Am. J. Pathol. Vol. 157, No. 2, 623-636); (b) shows the neuropathological staging proposed by Braak; (c) shows that the appearance of clinical dementia by DSM-IV criteria appears to correspond statistically to the transition between stages III and IV.

Figure 3 shows a schematic representation of a neurofibrillary tangle (top), and the immunoreactivity changes that are observed during disease progression (bottom). Taken from Bondareff *et al.* (1994) J. Neuropathol. Exp. Neurol. 53, 2, 158-164.

Figure 4 shows the chemical structures of: the minimum critical structure which enables high affinity binding to aggregated tau molecules (compound 4a); a biotinylated version of compound 4a (compound 4b); and an R-substituted derivative of compound 4a (compound 4c), wherein R is any suitable substituent.

Figure 5 shows the chemical structures of: primulin (compound 1a); thioflavin-T (compound 1b); 2-(4-amino phenyl)-6-methyl-1-sulphonate benzothiazole (compound 2); thiazin red (compound 3a); and thiazin yellow (compound 3b).

Figure 6 shows the fluorescence peaks of primulin in solution (left), and when bound within a pure preparation of PHFs (right).

Figure 7 shows the fluorescence peaks of primulin bound to PHFs, in the absence (left) and presence (right) of citraconic anhydride. As is shown, citraconic anhydride has the effect of disrupting the orderly structure of PHFs and releasing free tau and free unbound primulin.

Figures 8a and 8b illustrate exemplary tau-tau binding inhibitors, as disclosed in WO 96/30766.

Figure 9 shows the fluorescence of primulin bound to PHFs in the presence of alcian blue. The figure demonstrates that, in the presence of alcian blue, a disrupter of PHF structure (Ref 33), there is no disturbance of the characteristic bound primulin fluorescence peak at 460nm.

Figure 10 shows the effect of various compounds (MR′ molar ratio of compound:tau) on tau-tau binding.

Figure 11 shows schematically the synthesis of [^{11}C]-labelled methylene blue.

Figure 12 shows schematically the synthesis of [^{11}C]-labelled Azure B.

Figure 13 shows schematically the synthesis of a [^{18}F]-labelled derivative of compound 4a of Figure 4.

Figure 14 shows the dimensions, as indicated, of primulin, a benzothiazole analogue (denoted 'analog'), and 'thiazin yellow'.

Figures 15 and 16 illustrate the crystal structure of the 'B' part of the primulin structure (Soon-Beng Teo *et al.*, 1995, Acta Crystallogr., Sect. C, 591.

Figures 17 and 18 illustrate the crystal structure of a compound denoted N2A (Gilardi, R.D., 1972, Acta Chrystallogr., Sect. B, 107).

Figures 19 and 20 show the crystal structure of a diazoaminobenzene (Gladkova & Kondrashev, 1972, Kristallografiya (41) 17 33.

Figures 21 and 22 illustrate how the molecule of Figures 15 and 16 crystalises in space.

Figure 23 tabulates the minimum, maximum and mean dimensions for primulin, "analog", thiazin yellow, and the benzothiazole unit alone (i.e. structures 1b and 2 as shown in Figure 5). The corresponding methylene blue dimensions are given as 'MBCC' (carbon to carbon) and 'MBNN' (nitrogen to nitrogen).

Figure 24 shows a comparison of the mean, maximum and minimum extents of molecules which are active ligands (primulin and "analog"), and thiazin yellow (which is inactive as a ligand). The dimensions are given in angstrom units (AU).

Figure 25 shows a similar comparison for the basic benzothiazole nucleus (i.e. molecules 1b and 2 of Figure 5) and the diaminophenothiazines. These distances are carbon-to-carbon distances.

Figure 26 shows probability of extracellular tangles as a function of Braak staging. Stages 2 - 4 can be clearly distinguished from stage 1 on the basis of probability of extracellular tangles in E2/Trans and E4/HC.

Figure 27 shows quantity of aggregated tau protein in the extracellular space expressed in μg/g of brain tissue as a function of Braak staging.

The data was calculated as described in the materials and methods.

Figure 28 shows probability of intracellular tangles as a function of Braak staging. Intracellular tangles provide a poor basis for discrimination of early stages in these regions; but a good basis for discriminating stages 4 and 5 using neocortical regions.

Figure 29 corresponds to Figure 26, but wherein cases with MMSE scores greater than 21 in the 12 months prior to death were selected. Similar results are obtained

Figure 30 corresponds to Figure 28, but using cases with MMSE scores greater than 21 in the 12 months prior to death were selected. Similar results are obtained

Figure 31 shows extracellular tangle densities (counts per mm^2) as a function of Braak staging. Similar results are obtained to those shown in Figure 26 and 27.

Figure 32 shows intracellular tangle densities (counts per mm^2) as a function of Braak staging.

Figure 33 shows a barely visible tangle visualised with thioflavin-T at 0.001% (arrowhead). In ifI suspensions such as this, tangles can be seen by blue fluorescence which is not distinct from that associated with binding of contaminants in the preparation. The bottom panel shows that blue tangle fluorescence produced by thioflavin-T at 0.001% is displaced by yellow tangle fluorescence produced by primulin at 0.001%.

Examples

Methods and materials

PHF-binding compounds

Compounds used herein were supplied by ICI Pharmaceuticals unless stated otherwise. Thioflavin-T and thiazine yellow were purchased from Fluka AG.

Quantitation of Fluorescence

Serial 16 μm sections are cut from the hippocampus of a case dying with clinically and neuropathologically confirmed AD. These sections were stained with thioflavin-S at concentrations 0.01%, 0.001% or 0.0001% in water for 5-10 min, then washed in water, and mounted in Apathe's aqueous medium. In a second series of experiments, sections were cut from the hippocampus and nucleus basalis of Meynert. These sections were stained with primulin at concentrations 0.1%, 0.01%, 0.001% and 0.00001% in water for 5-10 min, then washed in water, and mounted in Apathe's aqueous medium.

A Leitz fluorescence microscope fitted with a photo-multiplyer tube (Model MPV-2) was used to quantitate fluorescence emission. Three Leitz filter blocks were used as follows:

1. Filter block H2, code 513 417
   Excitation range Band pass 390-490nm
   Mirror RKP 510 (i.e. transmit below 510 nm)
   Suppression filter LP 515 (i.e. reflect above 515 nm)

3. Filter block G, code 513 416
   Excitation range Band pass 350-460 nm
   Mirror RKP 510 (i.e. transmit below 510 nm)
   Suppression filter LP 515 (i.e. reflect above 515 nm)

4. Filter block A, code 513 410
   Excitation range UV band pass 340-380 nm
   Mirror RKP 400 (i.e. transmit below 400 nm)
   Suppression filter LP 430 (i.e. reflect above 430 nm)

Preparation of if I and II

IfI material was prepared as described by Wischik et al (1985) J Cell Biol 100: 1905-1912.

IfII material was prepared as described by Wischik et al (1995) Neurobiol Aging 16: 409-431. For experiments involving non-pronase digested ifII an identical protocol was followed, omitting the pronase digestion step.

Spectrofluorimetry of ifII

These measurements were carried out in a Perkin-Elmer spectrofluorimeter (model MPF-3). A concentration of ligand of 0.00001% was routinely used for all measurements. Primulin was found to have an excitation peak at 370 nm and an emission peak at 515 nm. All measurements were therefore carried out at a standard excitation wavelength of 370 nm, and a constant slit width of 3 mm.

Competitive Binding Assay

IfI material was homogenised in a 0.2 ml glass homogeniser in PBS. To the suspension, test compounds were added to final concentrations ranging from 0.1% to 0.00001%. These were allowed to incubate for 5 min and primulin was added at equivalent or lower concentration. The suspensions were transferred to a glass slide, and examined by fluorescence microscopy across a range of fluorescence filter blocks, covering excitation and emission wavelengths between 380 nm and 570 nm. The end point sought in these observations was displacement of typical primulin fluorescence from tangle fragments.

Ligand Electronmicroscopy

PHFs derived from an ifI fraction were deposited on a carbon coated grid after pronase digestion, and incubated briefly with a preparation of biotinylated Primulin, and then incubated with an anti-biotin antibody that has been conjugated with colloidal gold by the method of Slot and Gueze (1981).

Succinylation and Chromatography of IfII

Washed ifII fractions were taken up in 8 M urea/50 mM borate (1 ml, pH 9) and sonicated, 1 ml succinic anhydride in acetone was added to a final concentration of 250 mM succinate in 4 ml, and the pH was maintained at 8.5 with sodium hydroxide. The solution was clarified by centrifugation and applied to a Sephacryl S200 column equilibrated bicarbonate. The column eluate was monitored at either 230 or 280 nm.

Because succinylated fractions could not be visualised by Coomassie staining or silver staining of gels, bands were detected by autoradiography after specific chemical labelling of ifII fractions with

Bolton-Hunter reagent (Amersham).

For photoaffinity labelling of PHF derived peptides, ifI or ifII fractions were pre-incubated with an I^{125}-labelled photolabile derivative.

The photolabelled fraction running at a Kav of 0.21 was concentrated by ultrafiltration through an Amicon YM2 membrane (10ml), digested with chymotrypsin (0.01 mg/ml) in 50 mM ammonium bicarbonate. Chymotryptic fragments for sequence analysis were isolated by Dr H.C. Thogersen by reverse phase HPLC using a C18 column, with a 0-100% acetyl nitrile gradient, with 0.1% trifluoroacetic acid. The sequencing of chymotryptic peptides was carried out as described in Chapter 5.

Morphological Studies of PHFs in the presence of phenothiazines

For these experiments, ifII fractions were prepared as described above for electron microscopy. This material was either incubated directly with preparations of phenothiazines at final concentrations ranging between 0.1% and 0.0001% and then applied to carbon coated grids, and examined directly after LiPTA staining (1%). Alternatively, ifII suspensions were deposited on carbon coated grids, partially dried, and washed with solutions of phenothiazine. Such preparations were either stained directly with LiPTA or were processed further for immunoelectron microscopy using 6.423 as the primary antibody. Electron micrographs were recorded at nominal magnifications between 25,000 and 45,000.

Calculation of aggregated tau protein in the extracellular space expressed in μg/g of brain tissue as a function of Braak staging

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Previously reported PHF-tau levels in pmol/g (P) and tangle counts per mm^2 (T) in a clinically and neuropathologically staged cohort (R.Y.K. Lai, et al., *Neurobiol Aging* 16, 433 (1995)) were used to derive an estimate of PHF-tau level per affected pyramidal cell (PC) in pg/cell using the same ELISA in human brain. The tangle count per mm^2 provides an estimate of the number of affected pyramidal cells within a volume 1 mm x 1 mm x 0.1 mm (0.0001 cm^3), allowing that any tangle profile counted in a nominal 7 μm section could extend ~ 45 μm orthogonal to the section in either direction (S.M. Blinkov, I.I. Glezer, The human brain in figures and tables, a quantitative handbook; Plenum Press, NY, 1968, Table 204). The core PHF-tau level in pg/cm^3 is 10 x P since the PHF-core tau fragment is

10 kD (C.M. Wischik, *et al.*, *Proc. Natl. Acad. Sci.* USA 85, 4506 (1988)). From this, PC = (P x 10) / (T / 0.0001). At Braak stages 4-6 (H. Braak, E. Braak, *Acta Neuropathol.* 82, 239 (1991)), regional values for PC in grey matter were: frontal cortex, 0.13 ± 0.05 pg/cell; hippocampus, 0.60 ± 0.39 pg/cell; temporal cortex, 1.074 ± 0.44; entorhinal cortex 1.56 ± 0.63 pg/cell. These differences reflect anatomical differences, different regional rates of disease progression (C. Bancher, H. Braak, P. Fischer, K. Jellinger, *Neurosci. Lett.* 162, 179 (1993), also Gertz *et al.*, *Acta Neuropathol.* 95, 154 (1988)), and the degree to which tangle counts underestimate PHFs accumulating in dystrophic neuritis at more advanced stages of pathology (Lai et al, 1995, *loc cit*). The overall means provide an approximation for the PHF-levels per cell which would be relevant to AD. These are 0.37 ± 0.08 pg/cell for cases at Braak stages 1-3, and 1.08 ± 0.28 pg/cell for casea at Braak stages 4-6.

The specific data shown in Fig 27 was based on the following:

Number	BST	ME1T4	PC	PT4	REG3B	SE1T4
1	1.0000	0.3982	1.5600	0.6212	1.0000	0.3982
2	2.0000	6.7259	1.5600	10.4924	1.0000	2.7047
3	3.0000	14.9646	1.5600	23.3448	1.0000	2.9836
4	4.0000	33.6297	1.5600	52.4624	1.0000	10.9883
5	5.0000	44.3102	1.5600	69.1240	1.0000	13.0298
6	1.0000	0.0	0.6000	0.0	2.0000	0.0
7	2.0000	1.3865	0.6000	0.8319	2.0000	0.4531
8	3.0000	3.7169	0.6000	2.2302	2.0000	0.8060
9	4.0000	8.9384	0.6000	5.3630	2.0000	3.0048
10	5.0000	23.9479	0.6000	14.3687	2.0000	4.0567
11	1.0000	0.0	0.6000	0.0	3.0000	0.0
12	2.0000	0.0	0.6000	0.0	3.0000	0.0
13	3.0000	0.0	0.6000	0.0	3.0000	0.0
14	4.0000	0.1293	0.6000	0.0776	3.0000	0.1293
15	5.0000	2.2007	0.6000	1.3204	3.0000	1.0634

wherein:

BST is Braak Stage
ME1T4 is the extracellular tangle count
PC is an estimate of the PHF-tau concentration per cell (calculated as

above)
PT4 is the PHF content ascribed to extracellular tangles (PC x MEIT4)
REG3B is the grouping of brain regions into 3 groups as per Figs 26 and 27 of the SE1T4 is the standard error of the extracellular tangle count

Example 1 - aggregated tau in Braak staging

Based on immunochemical properties (Refs 26, 27, 30), it is possible to distinguish intracellular tangles from extracellular tangles. Both frequency of cases with tangles in these categories (ie probability) and their quantity (ie counts per mm^2) were determined in a prospective case series and grouped into the regions known to represent stages in the progression of pathology according to the system of Braak and Braak

As shown in Figure 26, stages 2 - 4 can be clearly distinguished from stage 1 on the basis of probability of extracellular tangles in E2/Trans and E4/HC. Also shown are the figures for F/T/P regions (neocortical regions - frontal, temporal, parietal).

Conversely, intracellular tangles provide a poor basis for discrimination of early stages in these regions, but a good basis for discriminating stages 4 and 5 using neocortical regions. Similarly, when cases with MMSE scores greater than 21 in the 12 months prior to death were selected, similar results were obtained. Again, similar results were obtained when tangle densities were determined.

These results can be converted into approximations for the quantity of aggregated tau protein in the extracellular space expressed in µg/g of brain tissue as described in Materials and Methods above. The results are shown in Figure 27. These are underestimates, as the tangle counts underestimate the quantity of aggregated tau protein.

In summary, these results demonstrate that extracellular deposits of PHF-tau in medial temporal lobe structures provide a basis for empirical staging of the neurofibrillary degeneration of AD. Such staging could only be accomplished by radio-imaging methods provided suitable ligands could be created.

Example 2 - assessment of compounds binding within the aggregated repeat domain of PHF-core tau protein

A prototype compound was obtained as one component of the crude, commercially-available preparation of thioflavin-S was separated into ~20 distinct constituents by analytical thin-layer chromatography, and preparative chromatography. Tests showed that not all of the constituents were able to act as effective tangle ligands. Specifically, pure primulin (Figure 5, compound 1a) was found to label tangles, but the benzothiazole thioflavin-T (Figure 5, compound 1b) was much less effective, although it labelled amyloid preferentially.

Furthermore, compound 1a was found to displace compound 1b at tangles when the latter was introduced at 10-fold excess into crude tangle extracts.

A possible difference was postulated to be the sulphonate group at position 1 (Figure 5, compound 2 [2 - (4 - amino phenyl) - 6 - methyl - 1 - sulphonate benzothiazole]). However, primulin (compound 1a) was found to displace this from tangles (though not amyloid). Therefore, tangle labelling is not due solely to the sulphonated benzothiazole structure, indicating that a longer aromatic structure is required.

Purified thiazin red (Figure 5, compound 3a) was found to compete with primulin at equivalent concentrations, whereas the compound 3b (thiazin yellow, Figure 5) did not. Therefore, an extended aromatic benzothiazole structure does not, *per se*, determine high binding affinity within tangles.

In order to define a minimum critical requirement for competitive binding, the sulphonated benzothiazole was extended by addition of a single phenyl group across a diamino-linkage. This compound (Figure 4, compound 4a), although not fluorescent, was found to compete out thiazin red and primulin fluorescence at equivalent concentrations. Compound 4a therefore defines the minimum critical structure required for high affinity binding within the tangle.

In order to prove that the binding site within the tangle was in fact the PHF itself, compound 4a was further extended with addition of a biotin group (Figure 4, compound 4b). Since this was still found to compete primulin and thiazin red, compound 4b preserved high affinity binding within the tangle. Furthermore, immunogold-conjugated anti-biotin

antibody was found to label isolated PHFs pre-incubated with compound 4b, whereas no labelling was demonstrated without pre-incubation or pre-incubation with biotin alone. Finally, when a photo-activated conjugate of the compound was prepared, it was possible to identify and sequence the labelled protein. This was found to be the same core tau fragment as that isolated from the core of the PHF, which comprises the repeat region of the tau protein.

In summary, these results demonstrate unequivocally that the binding site for compounds 4a and 4b is within the aggregated repeat domain of the tau protein of the PHF-core. Furthermore, they demonstrate that compound 4a can be used as a chelate for addition of functional groups without disturbing ligand activity within the PHF core. Therefore, compound 4a could be used as chelate for addition of technetium or other imaging moiety to generate a ligand suitable for detecting e.g. extracellular tangles in AD.

<u>Example 3 - determination of optimum dimensions of ligand molecules</u>

Figure 14 shows three of the structures described above, along with their dimensions as indicated. For example, the C11-C1 distance and C10-C1 distance are shown for primulin, a benzothiazole analogue (denoted 'analog'), and 'thiazin yellow'.

Figure 15 and 16 illustrate the crystal structures of the 'B' part of the primulin structure (Soon-Beng Teo *et al.*, 1995, Acta Crystallogr., Sect. C, 591. As can be seen from Figure 16, which is a 'side-on' view, the molecule is essentially flat, although it has a slight twist. The' A' part of primulin can be computed from the same molecule. From this, one can derive measures of A+B, which provide an indication of the actual length of one of the active species of the present invention.

To compute the size of the "analog" shown in Figure 14, measurement A was used from the data of Figure 15, and measurement B was determined from a molecule denoted N2A and shown in Figures 17 and 18 (Gilardi, R.D., 1972, Acta Chrystallogr., Sect. B, 107). As can be seen from the side-on view in Figure 18, this part of the molecule is completely flat. The same measurements apply to thiazin red, which is identical in its dimensions to the "analog".

The size of thiazin yellow (shown in Figure 14) was determined as follows. The 'A' part comes from the molecule of Figure 15 which was used for primulin, while the 'B' part comes from the molecule shown in Figures 19 and 20 (Gladkova *et al.*, 1972, Kristallografiya 41). Again, part B of the molecule is completely flat, and the only difference with respect to the molecule shown in Figure 17 is the distance between the aromatic groups.

Figures 21 and 22 illustrate how the molecule of Figure 15 crystallises in space. As can be seen, the molecule forms an alternating 'herring-bone' pattern, and does not stack. In comparison, the crystal structure of methylene blue indicates that the molecules form stacks with alternating sheets of water molecules between the pi-bonded stacks.

Figure 23 tabulates the minimum, maximum and mean dimensions for primulin, "analog", thiazin yellow, and the benzothiazole unit alone (i.e. structures 1b and 2 as shown in Figure 5). The corresponding methylene blue dimensions are given as 'MBCC' (carbon to carbon) and 'MBNM' (nitrogen to nitrogen).

Figure 24 shows a comparison of the mean, maximum and minimum extents of molecules which are active ligands (primulin and "analog"), and thiazin yellow (which is inactive as a ligand). The dimensions are given in angstrom units (AU). In Figure 25, a similar comparison is made for the basic benzothiazole nucleus (i.e. molecules 1b and 2 of Figure 5) and the diaminophenothiazines. These distances are carbon-to-carbon distances.

The above results illustrate that the molecules provided herein are substantially flat. There is, however, a fundamental difference in activity between ligands according to the present invention and other molecules discussed above. As is shown in the Figures, suitable ligands according to the invention comprise long, flat molecules with dimensions between 14.783 and 15.261 AU. On the other hand, a longer molecule, such as thiazin yellow, which exceeds these dimensions (mean 15.927 AU) does not serve as an effective ligand, even though it is flat. However, certain shorter, flat, molecules bind preferentially to amyloid.

Example 4 - comparison of ligand molecules and inhibitors

There appears to be a fundamental difference in activity of the molecules

which are effective ligands, compared with those which are effective inhibitors of tau-tau binding. The benzothiazole molecule does not disrupt PHFs, nor indeed do any of the ligands, whereas the diaminophenothiazine series constitute PHF-disrupters and tau aggregation inhibitors.

Further investigations into the relationship between aggregation-dependent tau ligands and tau aggregation inhibitors were carried out using primulin. Primulin in solution has a fluorescence peak at 520 nm. This shifts to 470 nm when primulin is bound within a pure preparation of PHFs (Figure 6). Treatment of PHFs with citraconic anhydride, which has been shown to disrupt the structure of the PHF and liberate free tau, was found abolish the 470 nm fluorescence peak (Figure 7). Therefore, binding by such compounds is dependent on the polymerised state of tau found in the PHF, but is not present in free tau.

Compounds have been identified which disrupt the structure of the PHF and reverse the proteolytic stability of the PHF core (see WO 96/30766). Examples of such compounds are shown in accompanying Figure 8. The present inventors have now identified that these compounds bind to tau at a specific binding site within the high affinity tau-tau binding domain. However, it is found that such compounds do not disrupt the binding of primulin to tau in aggregated tau molecules, as shown by the retention of the fluorescence peak of primulin at 470 nm (Figure 9). In this way, the present inventors have established that compounds which act as ligands of aggregated tau do not bind at the same site(s) as compounds which are tau-aggregation inhibitors.

This point was further examined by studying the potency of typical aggregated tau ligands as tau-aggregation inhibitors. It has been shown previously that tau aggregation inhibitors (e.g. diaminophenothiazines) can be identified on the basis of inhibition of tau-tau binding in a solid-phase assay (WO 96/30766). When tested in the same assay, primulin and thiazin red were found to be weak inhibitors of tau-tau binding (Figure 10). Thus, although these compounds are potent ligands for tau within the PHF-core, they are weak inhibitors at the site required for inhibition of tau-tau binding.

Demonstration that compounds of the diaminophenothiazine-like class bind tau in the aggregated state is provided by the direct demonstration of

disruption of PHF structure in the presence of sufficiently high concentrations, particularly of compounds such as methylene blue. Thus, compounds of the diaminophenothiazine-like class which are inhibitors of tau-tau binding can serve as aggregated-tau ligands at lower concentrations.

In summary, the inventors have found that it is possible to define two classes of binding site within the core-PHF tau aggregate. Both are potentially useful for the development of radiological imaging ligands:

(i) Sulphonated benzothiazole-like sites: compounds of this type, associated with suitable chelates such as technetium, may serve as ligands for extracellular tangles, due to their size and charge.

(ii) Diaminophenothiazine-like sites: such compounds, when suitably labelled with a positron-emitting functional group, would serve as ligands for all tau aggregates, and would be capable of crossing the blood-brain-barrier (Ref 36) and entering cells. Thus these compounds, and derivatives thereof, have potential use in the labelling of intracellular tangles, e.g. those present in the brains of AD patients, or intracellular tangles when used at lower concentration.

Example 5 - PET using ligand molecules and inhibitors

Figures 11 to 13 indicate typical synthesis methods which could be used to convert either the diaminophenothiazines or the "analog" into positron emitting species. The chemistry of the syntheses and general methodology are all familiar to persons skilled in the art. These examples are given without any implied restriction as to ultimate methodology.

Example 6 - example assays for developing novel diagnostic ligands

Having defined these two classes of ligands suitable for labelling PHFs in AD, further ligands can be developed using the compounds/derivatives in screening assays. Furthermore, modelling methods can be based on the ligands already presented.

(i) Identification of novel ligands at the sulphonated-benzothiazole site.

Using a suitably labelled preparation of a known sulphonated benzothiazole, incubated with a preparation of aggregated tau molecules (e.g. preaggregated tau in solution, or bound to a solid phase, or highly enriched PHFs isolated from AD brain - see PCT/EP96/01307) compounds suspected of being suitable ligands can be introduced, and their capacity to compete with the known ligand in such a way as to prevent binding within the PHF can be tested.

(ii) Identification of novel ligands at the phenothiazine site.

The tau-tau binding assay described in WO 96/30766 can be used as a preliminary screen to identify potential inhibitors at the tau-tau binding site. Likewise, a suitably-labelled preparation of known diaminophenothiazines, incubated with a aggregated tau as described above, could be used to screen for other compounds which are suspected of being competitors at this PHF-binding site and thus potentially suitable PHF ligands.

The physical implementation of competitive assays is well known in the art. It may include measurement of fluorescence, radioactivity or any other suitable reporting system which derives from sulphonated benzothiazole-like compounds or diaminophenothiazine-like compounds not bound to PHFs, i.e. those which remain in solution.

Example 7 - blocking ligands

Compounds such as thioflavin-T and -S strongly stain amyloid deposits. However Figure 33 demonstrates that such compounds can be displaced from tangles by primulin. Therefore these compounds may be used as blocking reagents to saturate binding sites which are not of interest without inhibiting the binding of ligands to aggregated tau.

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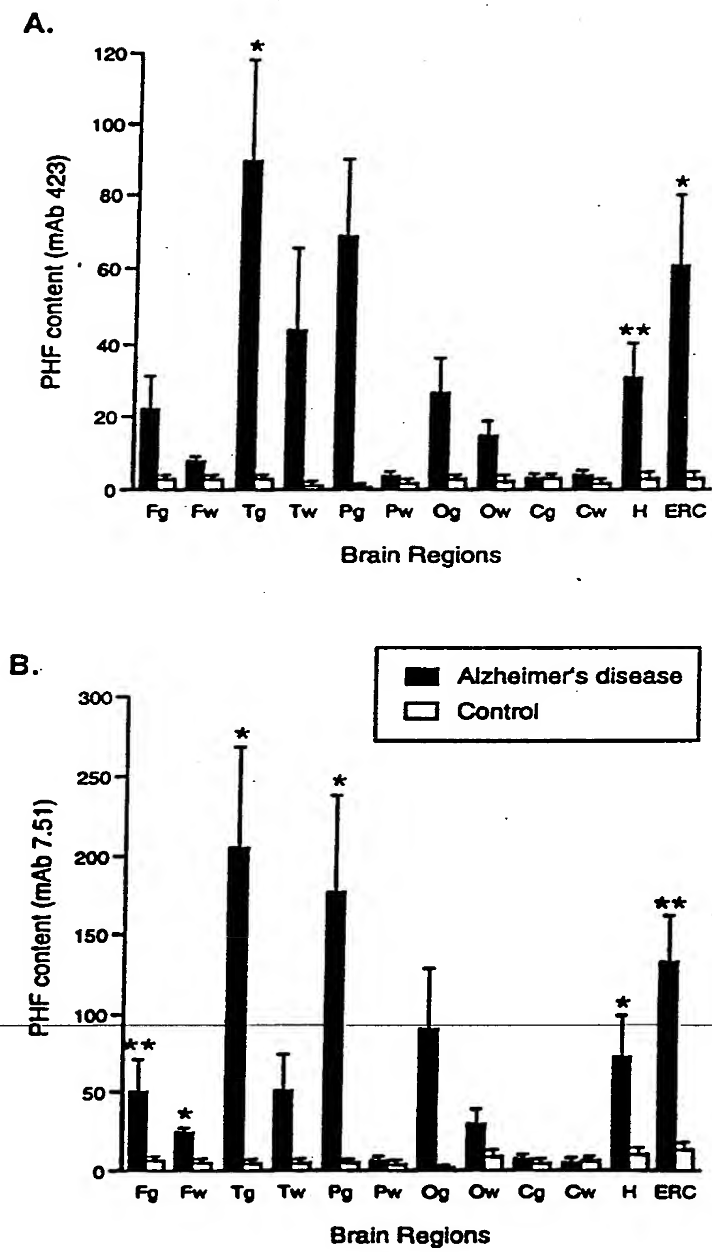

A.
PHF content (mAb 423)
120
100
80
60
40
20
0
*
*
**
Fg
Fw
Tg
Tw
Pg
Pw
Og
Ow
Cg
Cw
H
ERC
Brain Regions
B.
Alzheimer's disease
Control
PHF content (mAb 7.51)
300
250
200
150
100
50
0
Brain Regions

Figure 1

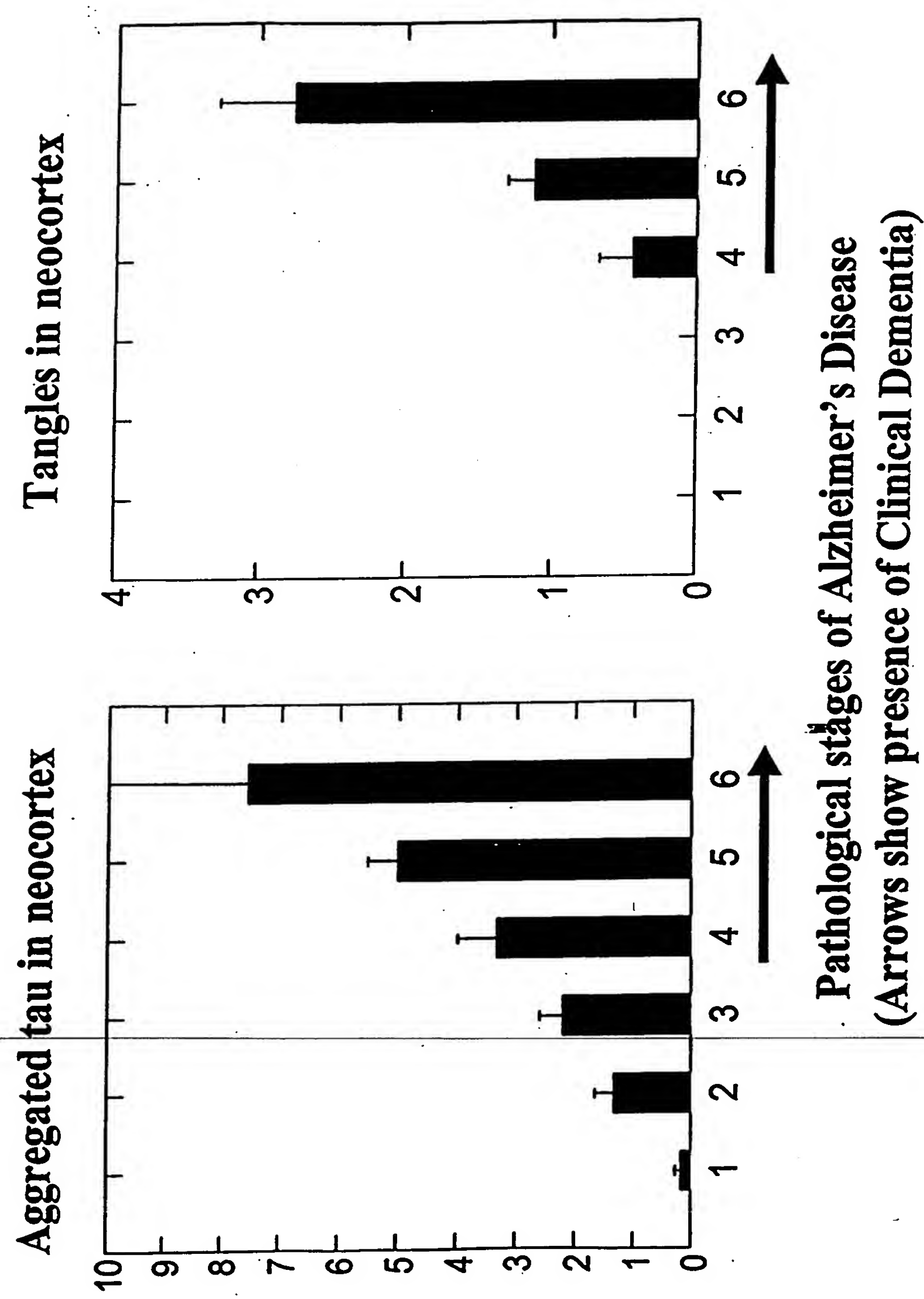

Aggregated tau in neocortex
Tangles in neocortex
Pathological stages of Alzheimer's Disease
(Arrows show presence of Clinical Dementia)
0
1
2
3
4
5
6
7
8
9
10

Figure 2

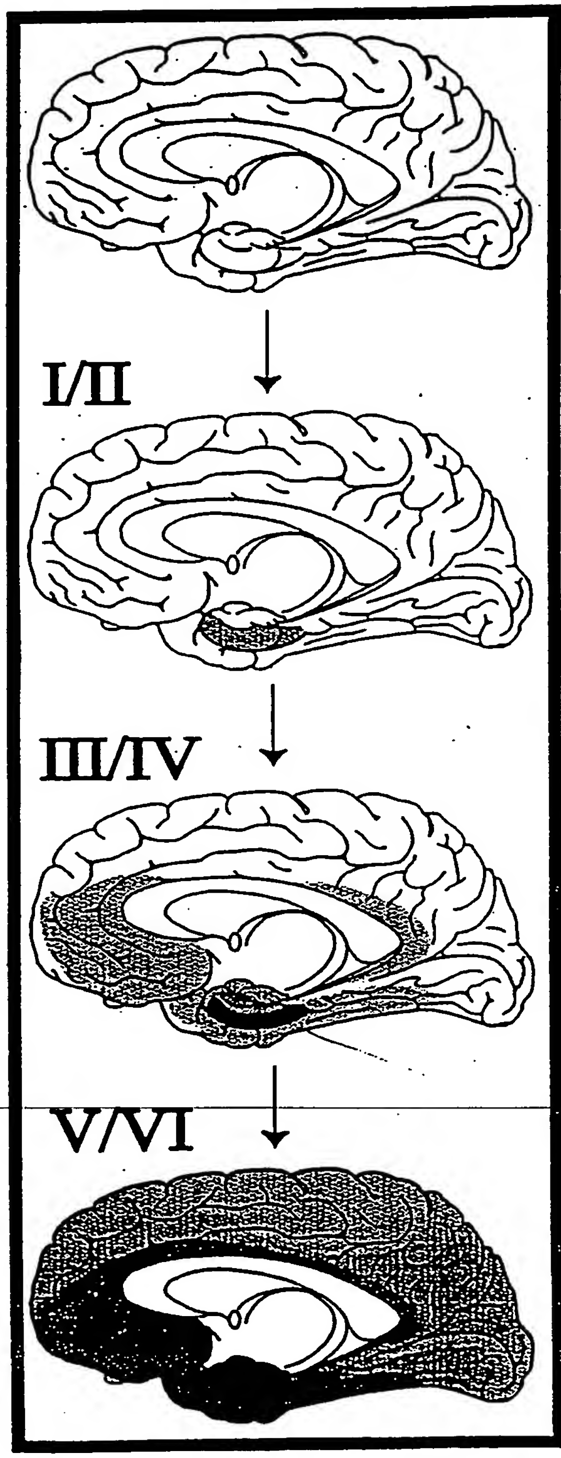


Figure 2b

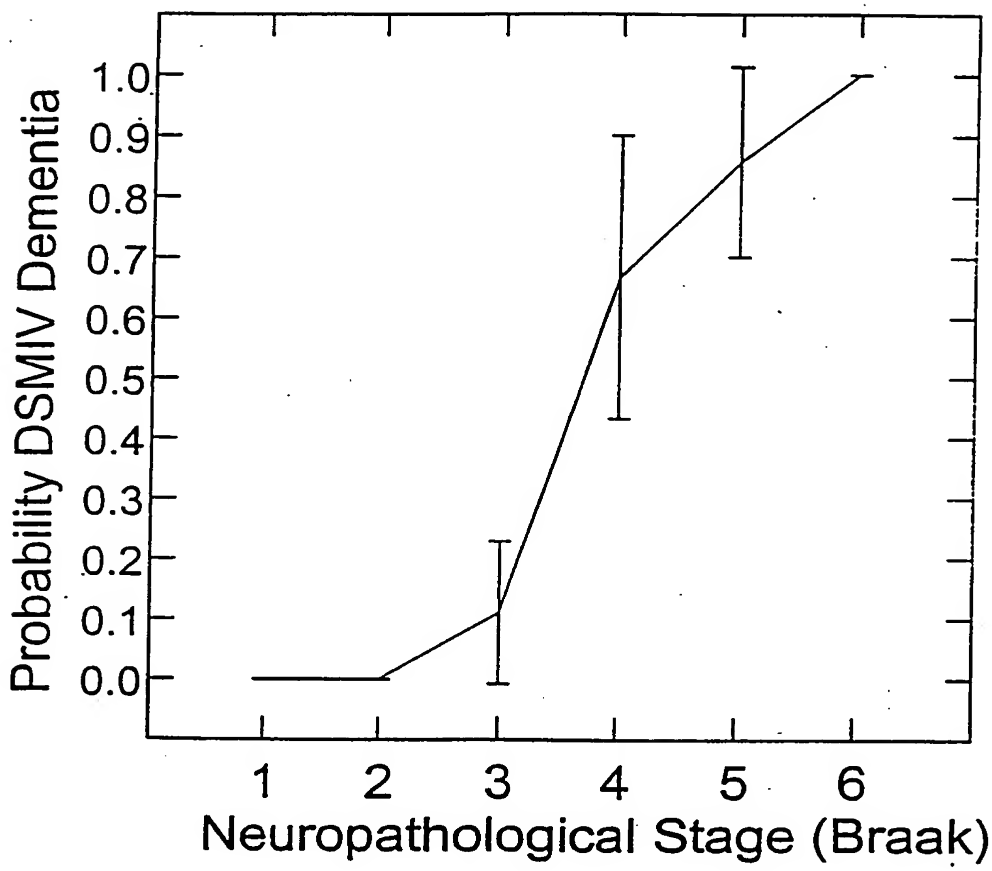
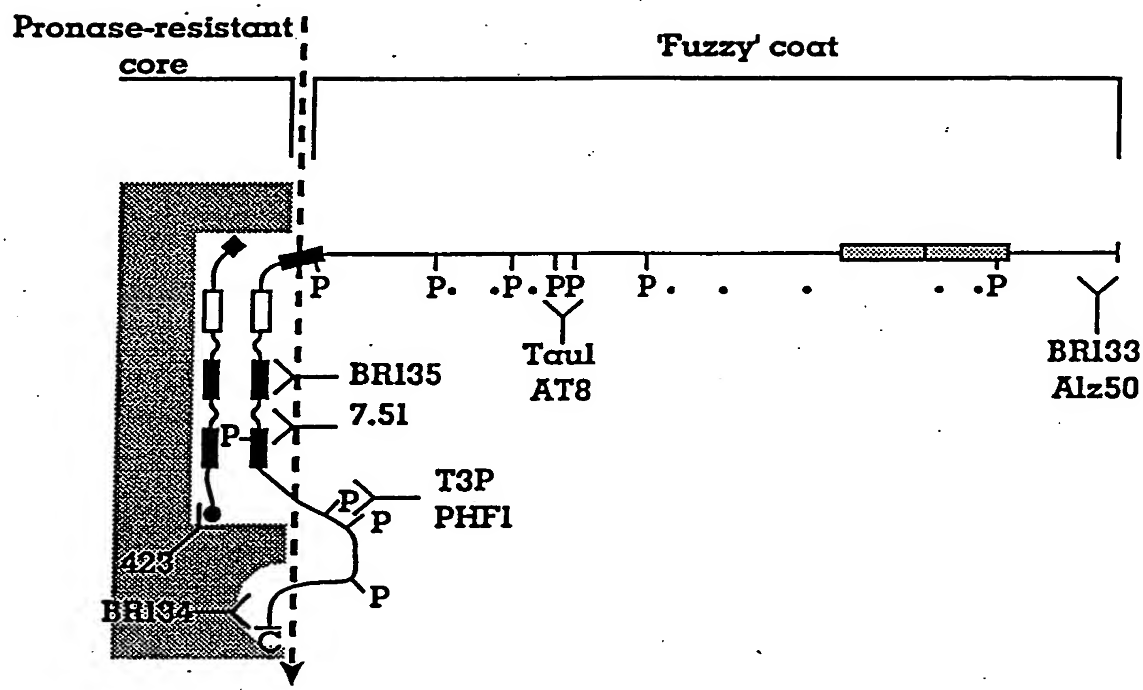

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Probability DSMIV Dementia
1
2
3
4
5
6
Neuropathological Stage (Braak)

Figure 2c

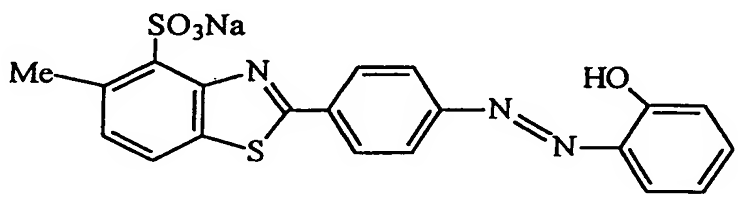


Antibody	Extracellular Stages 3	Intra-/Extra-Cellular Stages 2	Intracellular Stage 1
BR 133	-	-	+++
mAb 18.8	-	-	+++
BR 134	+	++	+++
mAb 11.57	+/-	++	+++
mAb 25.6E	+/-	++	+++
BR 135	+++	+++	+++
mAb 423	+++	+++	+

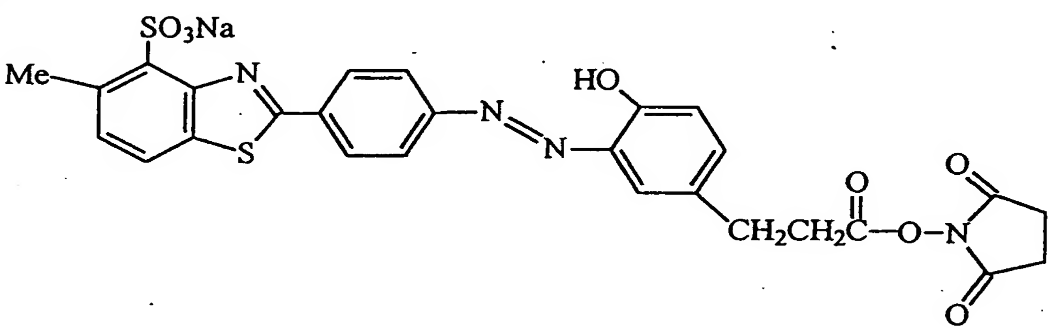
Figure 3

Best Available Copy

4a



4b



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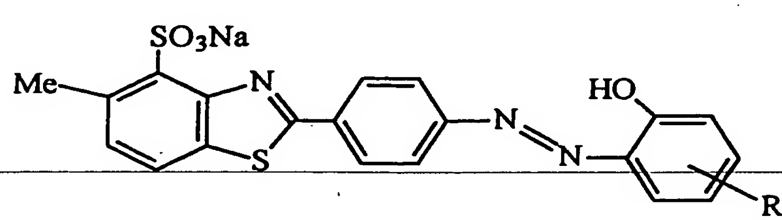
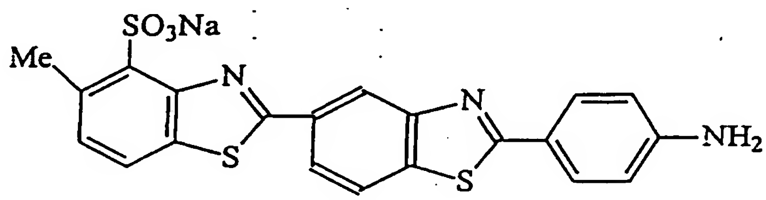
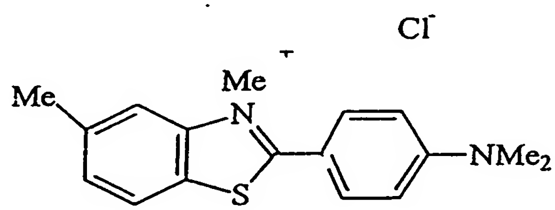


Figure 4

1a



1b



2

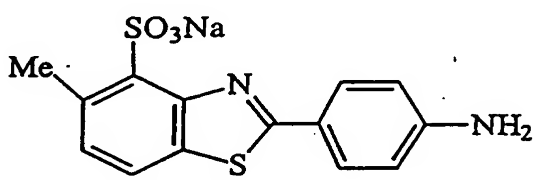
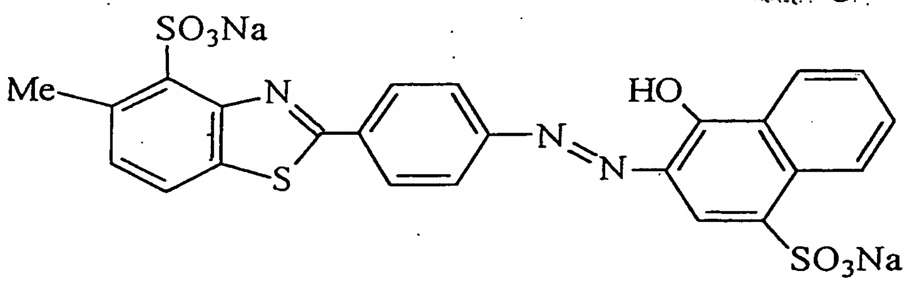


Figure 5

3a

thiazin red.



3b

thiazin yellow

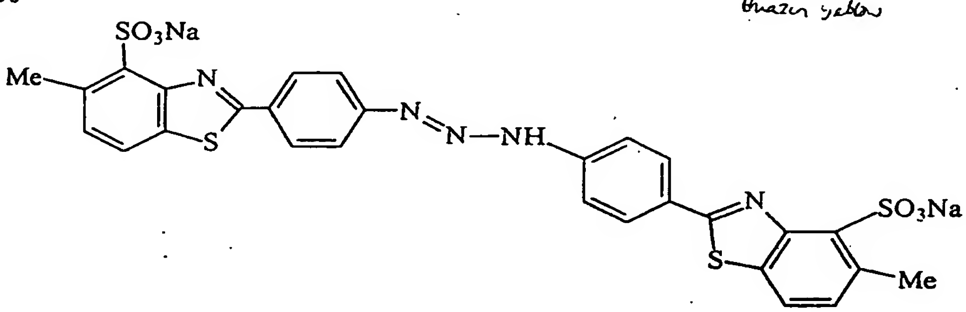
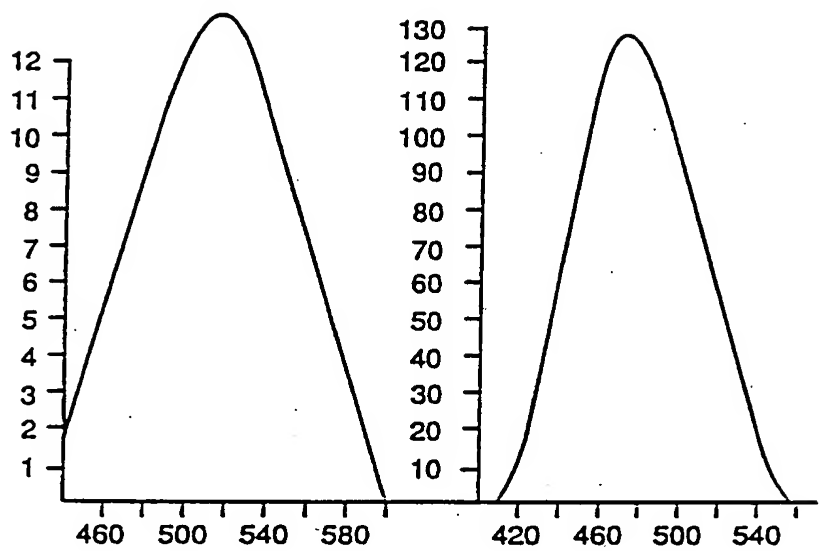


Figure 5 cont ...

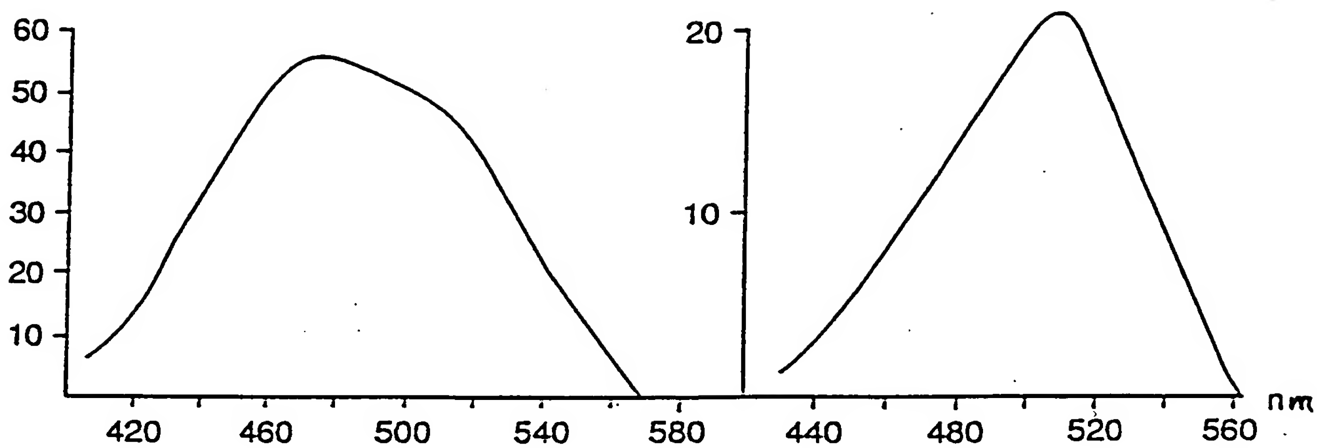


c14537
(0.00001%)

if II +c14537
(0.00001%)

(c14537 = primulin)

Figure 6



if II + c14537

(0.00001%)

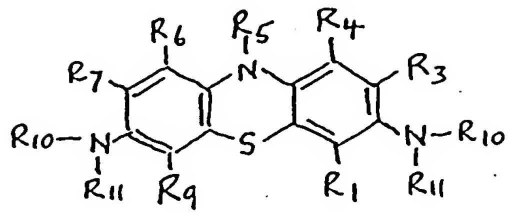
if II + 3 λC.A.

+ c14537 (0.00001%)

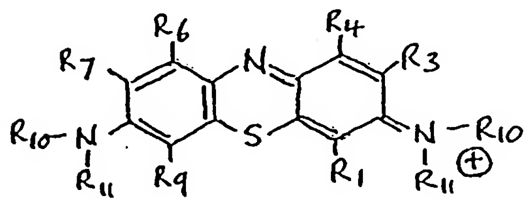
(c14537 = primulin)

Figure 7

(I)



(II)



(III)

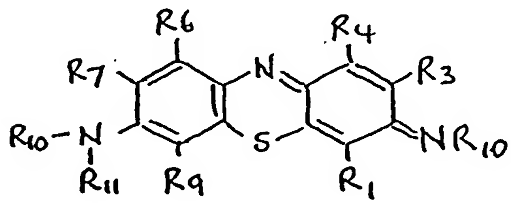
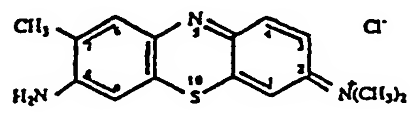


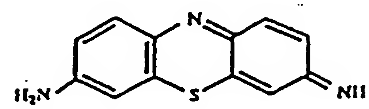
Figure 8a

Diamino phenothiazines with varying methyl groups

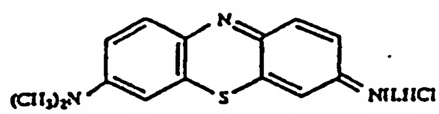
Tolonium chloride



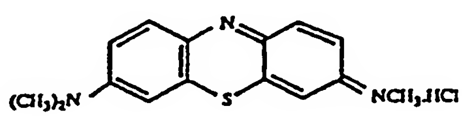
Thionine



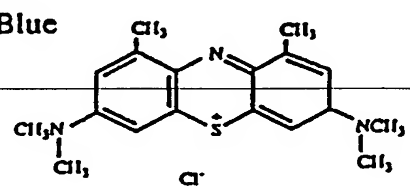
Azure A



Azure B



1,9-Dimethyl-Methylene Blue



Methylene Blue

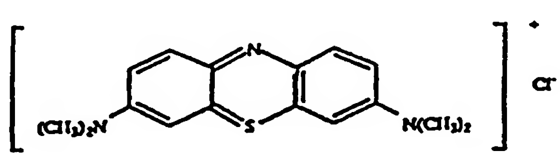
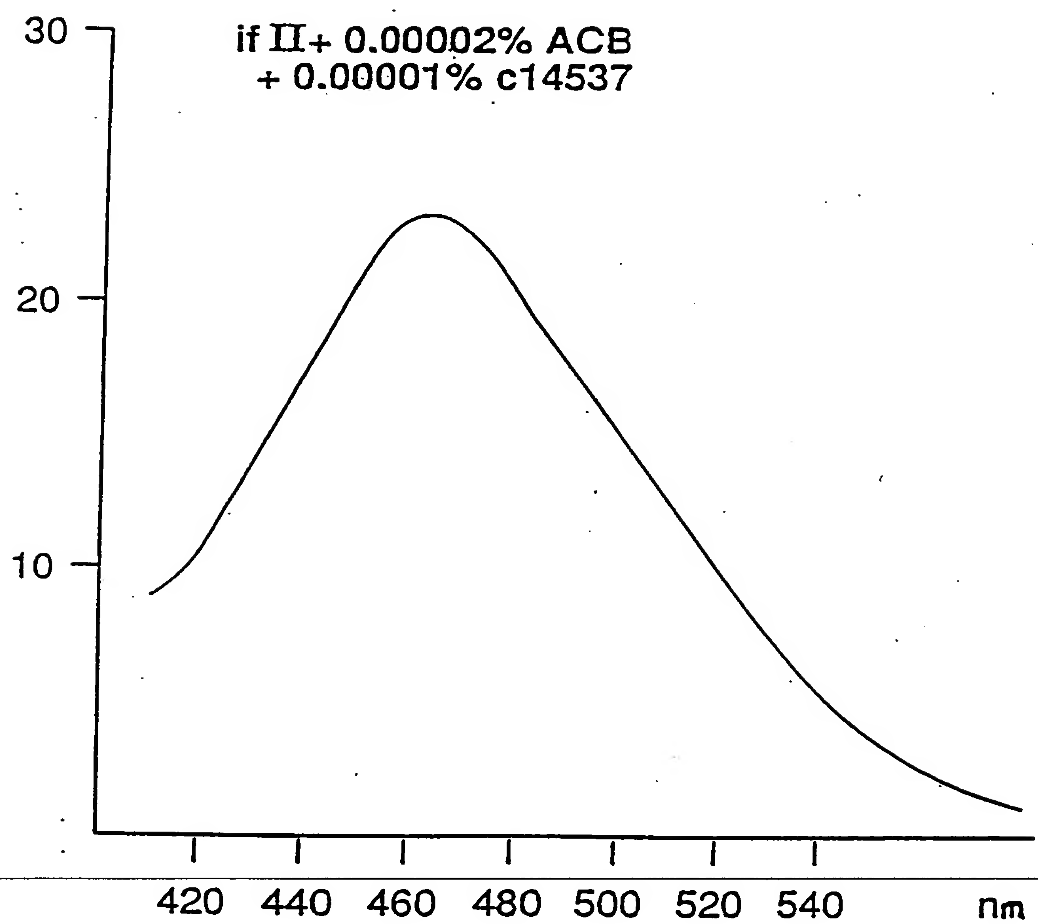


Figure 8b



(ACB = Alcian Blue, c14537 = primulin)

Figure 9

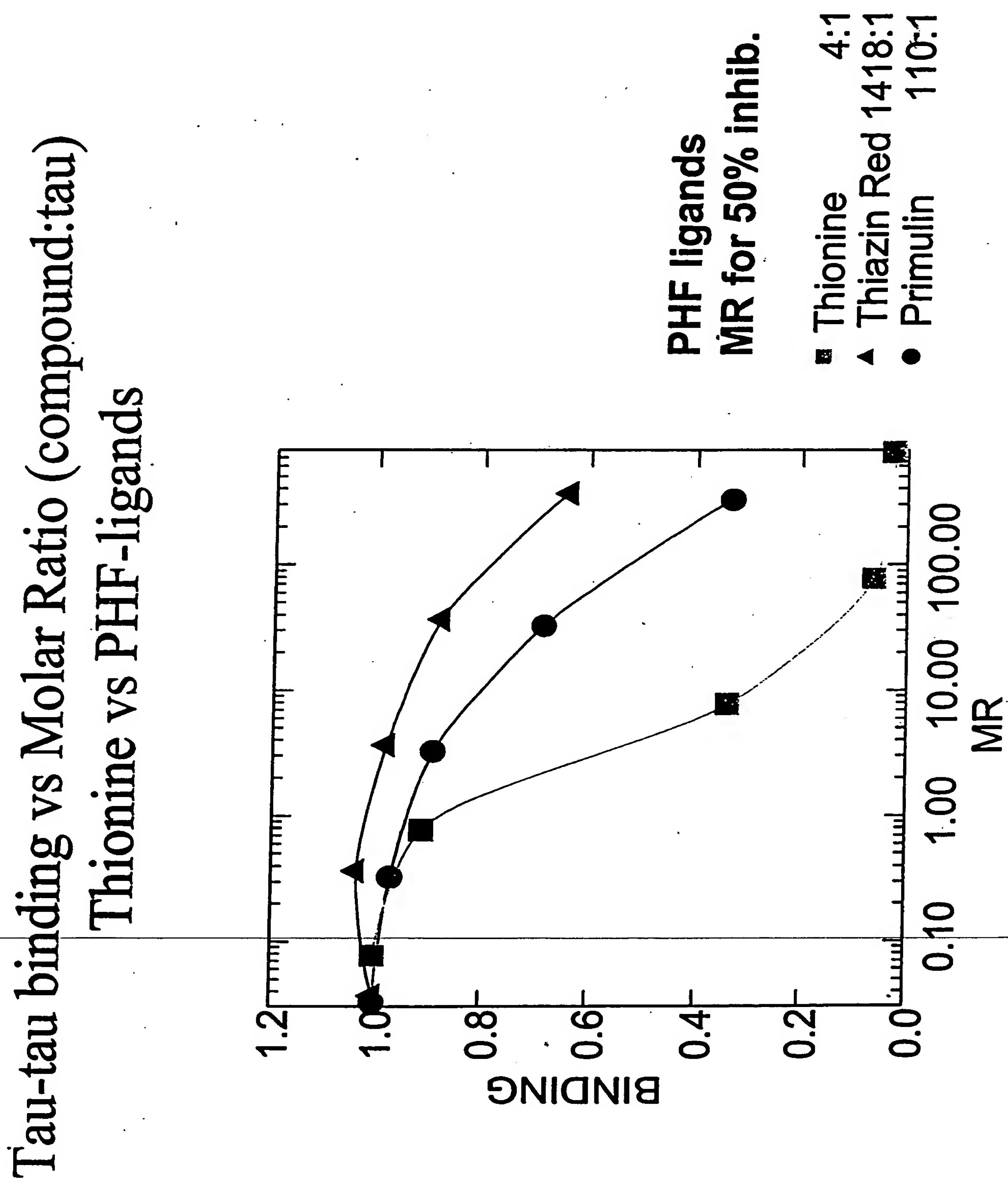

Tau-tau binding vs Molar Ratio (compound:tau)
Thionine vs PHF-ligands
BINDING
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.10
1.00
10.00
100.00
MR
PHF ligands
MR for 50% inhib.
Thionine 4:1
Thiazin Red 1418:1
Primulin 110:1

Figure 10

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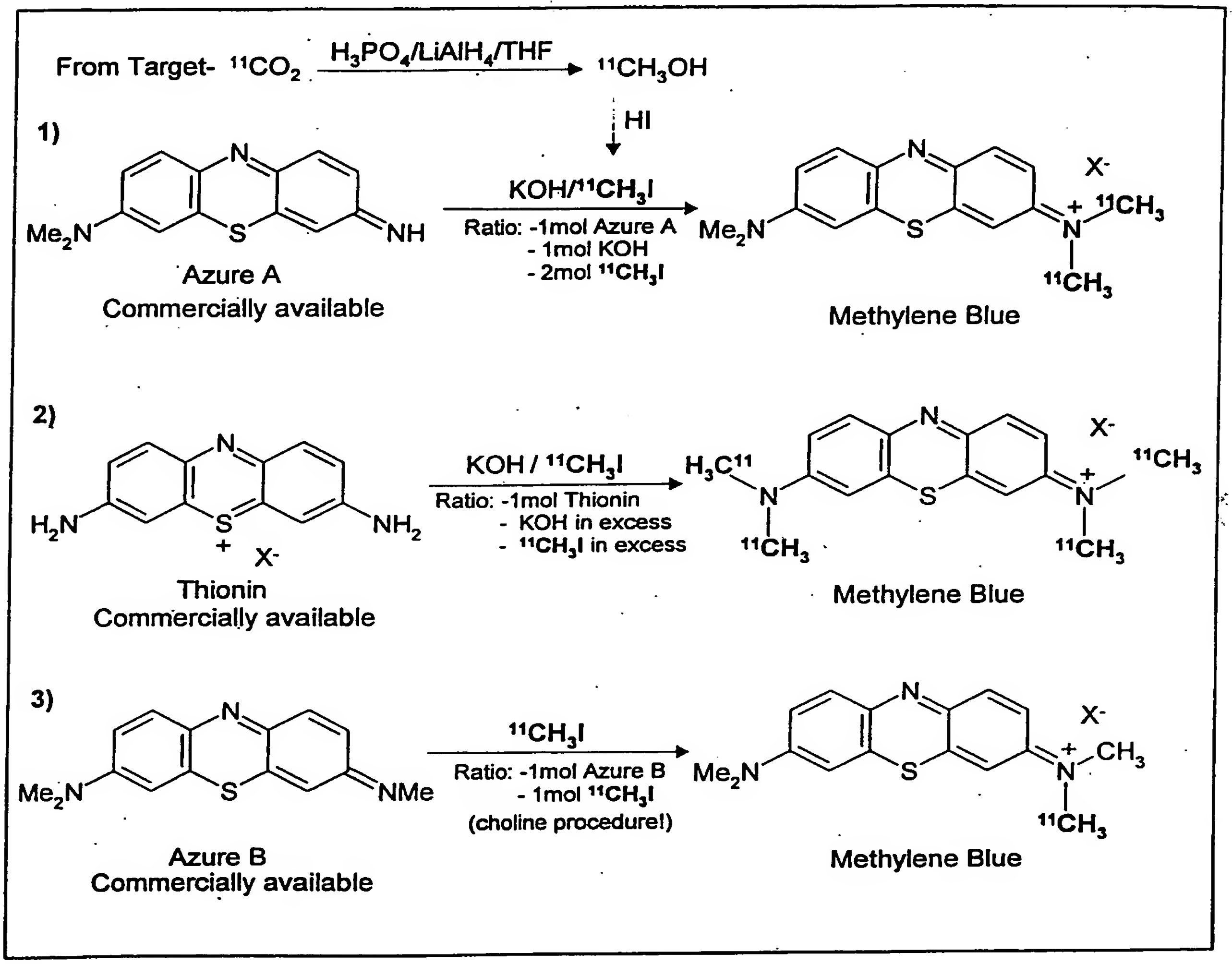
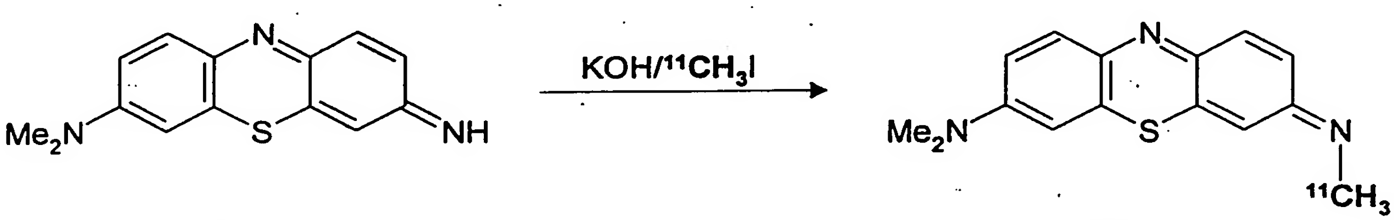
Synthesis of [^{11}C] labelled Methylene Blue



- **Reaction: Methylation of Amines**
- **N-Methylation with [^{11}C]Iodomethane**
- **Possible by-products can be separated via HPLC**

Figure 11

Synthesis of [^{11}C] labelled Azure B



Azure A
Commercially available

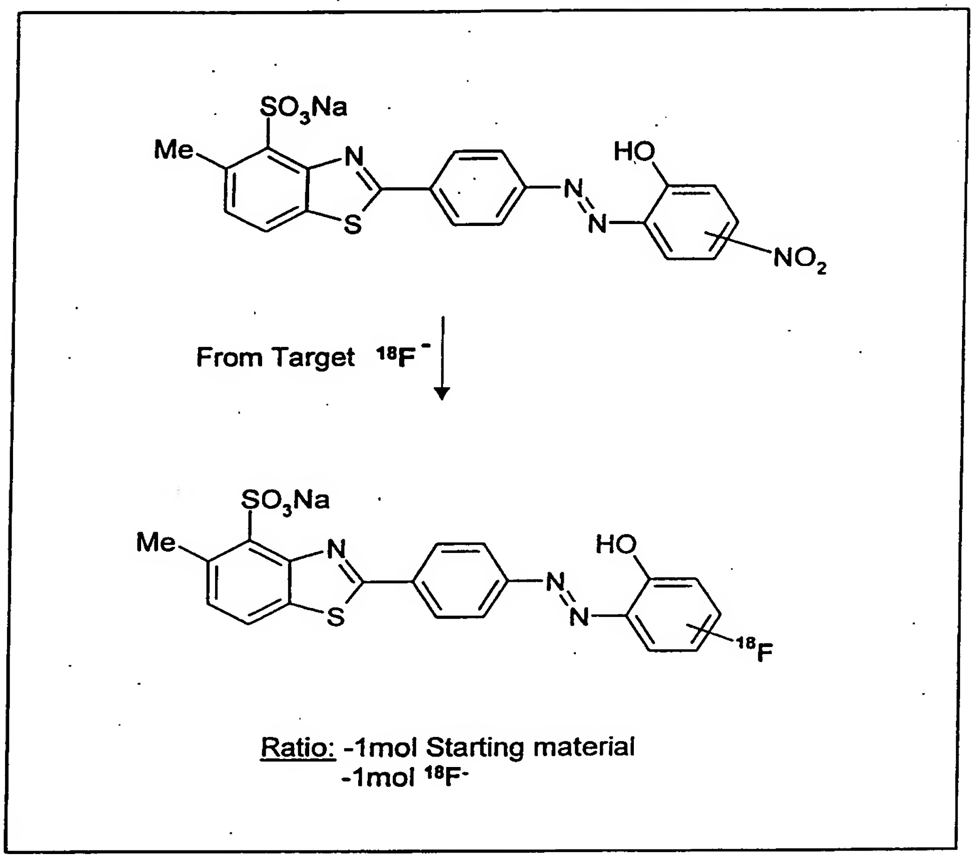
Azure B

Ratio: -1mol Starting material Azure A
-1mol KOH
-1mol $^{11}CH_3I$

- **Reaction: Methylation of Amines**
- **N-Methylation with [^{11}C]Iodomethane**
- **Possible by-products can be separated via purification cartridges and HPLC**

Figure 12

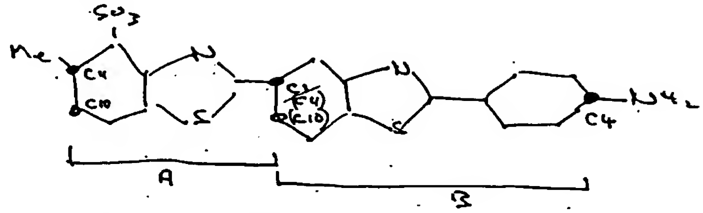
Synthesis of [^{18}F] labelled



- **Reaction: Nucleophilic aromatic substitution**
- **[^{18}F] Fluoro for Nitro exchange at the aromatic ring**
- **Precursor has to be synthesised**
- **Possible by-products can be separated via purification cartridges and HPLC**

Figure 13

Primuline.

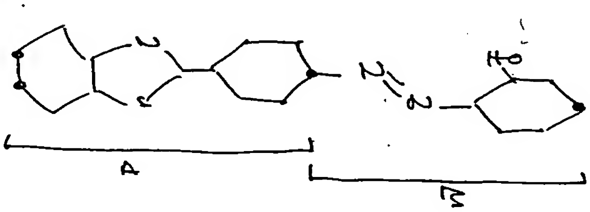


A. C11 – C1 6.013
C10 – C1 6.157

B C11 – C4 8.770
C10 – C4 8.855

A + B 14.783
14.968
14.927
15.112
[14.8475 ± 0.0677] (se)
0.1354 (sd)

Analog



A C11 – C4 8.770
C10 – C4 8.855

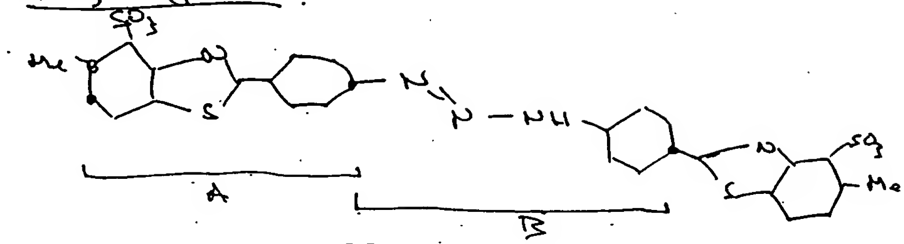
B C1_3 – C7 6.306
C7 – C4 6.280
C7_3 – C4_3 6.280
C7_3 – C1 6.306

15.076
15.050
15.050
15.076
15.261
15.261
15.235
15.235 15.1555 ± 0.0999 (sd)
0.0353 (se)

Figure 14

Best Available Copy

Thiazin-Yellow



A   C11 – C4   8.770
     C10 – C4   8.955
(185)

B   C7 – C5   7.164
     C7 – C4   7.001
     C9 – C1   7.000
     C10 – C1   7.183

     C7 – C5   6.881
     C7 – C4   7.115
     C9 – C1   6.957
     C10 – C1   7.117

15.934
15.771
15.770
15.953
15.751
15.835
15.727
15.887
16.119
15.956
15.955
16.138
15.936
16.070
15.912
16.072

15.9273 ± 0.1288 (sd)
          0.0322 (se)

Figure 14 cont …

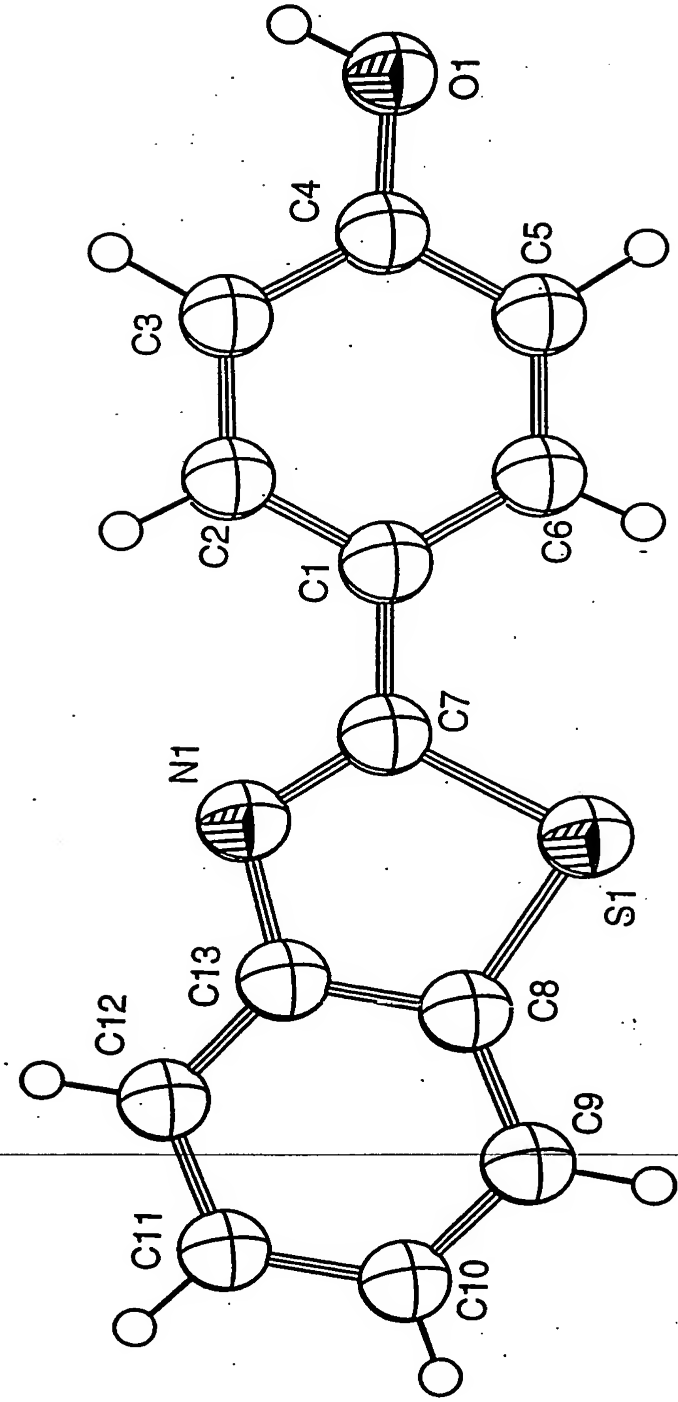


Figure 15

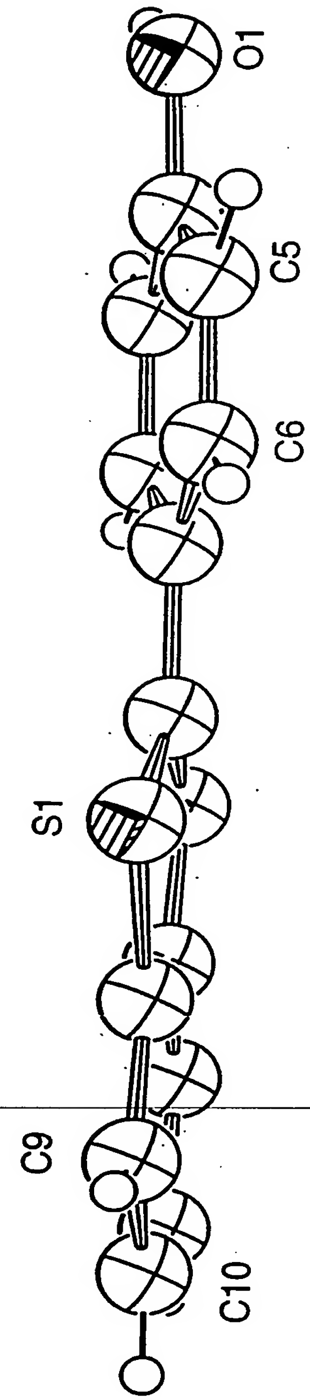


Figure 16

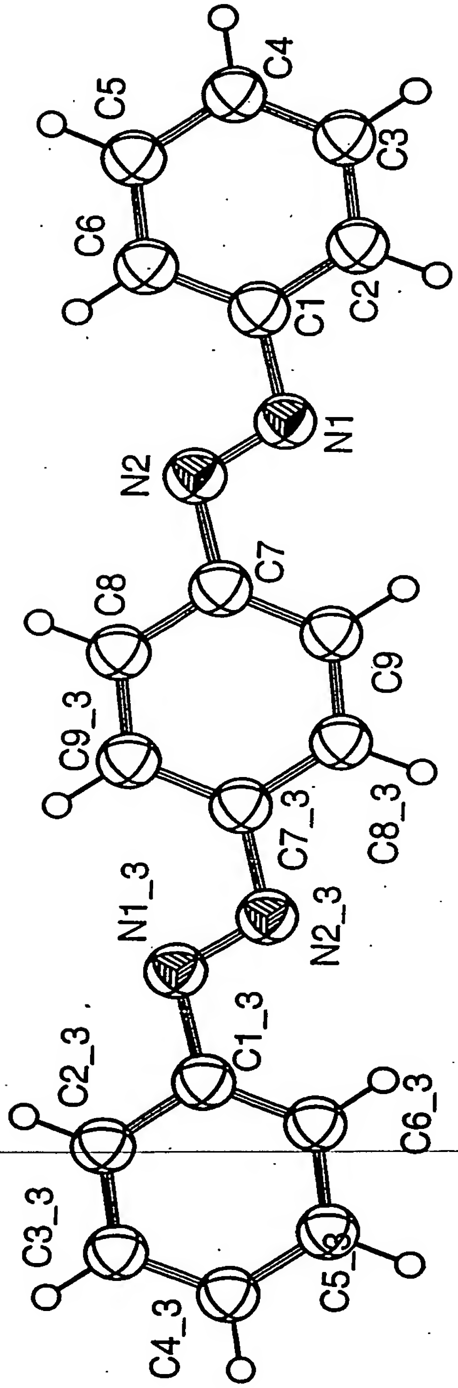

C4
C5
C3
C6
C2
C1
N1
N2
C7
C8
C9
C9_3
C8_3
C7_3
N1_3
N2_3
C1_3
C2_3
C6_3
C3_3
C5_3
C4_3

Figure 17

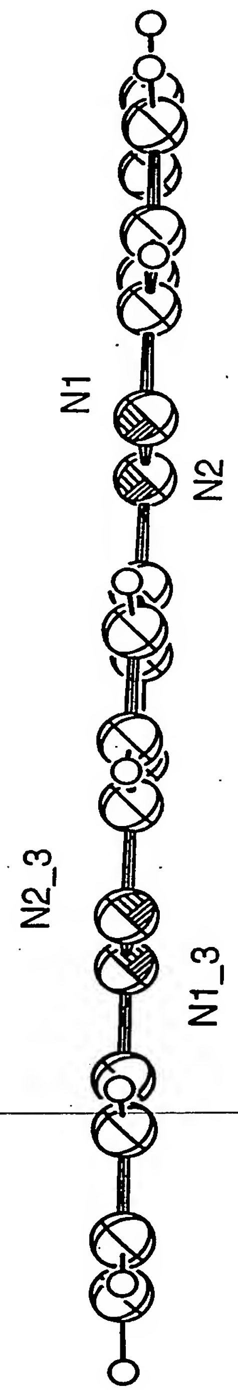


Figure 18

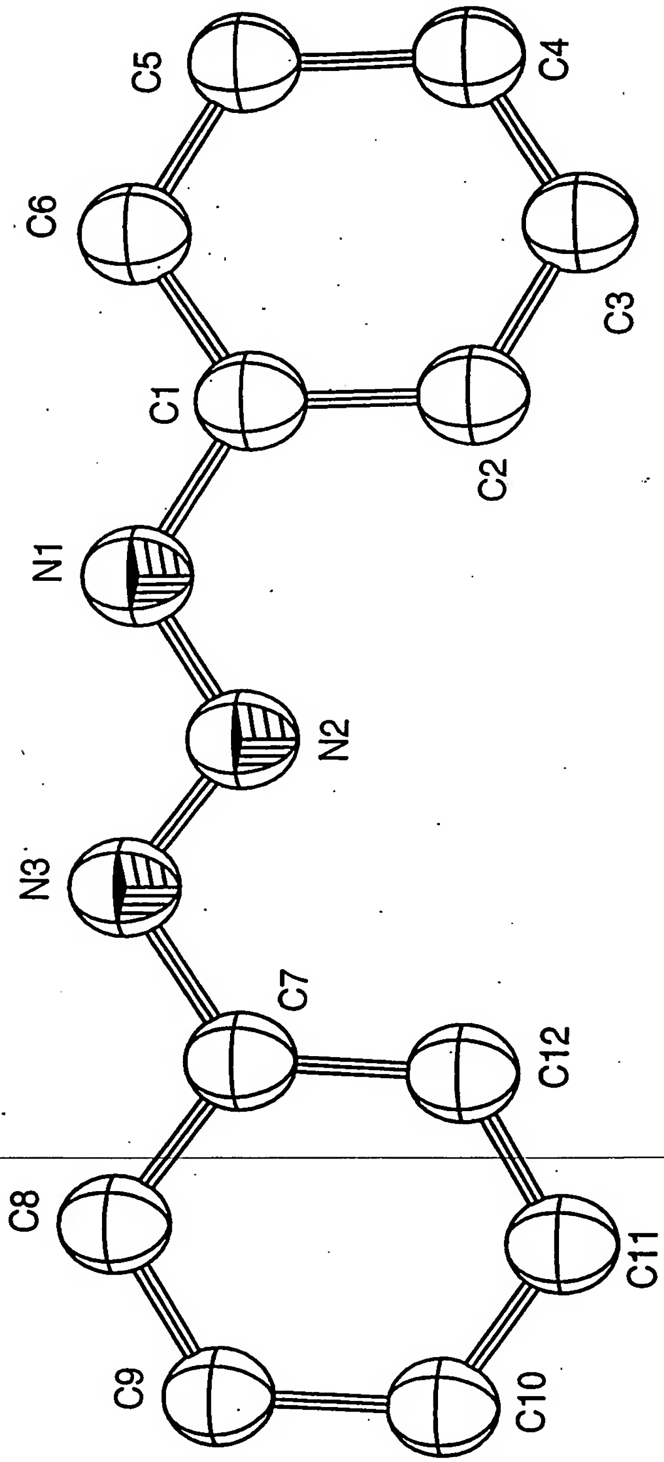


Figure 19

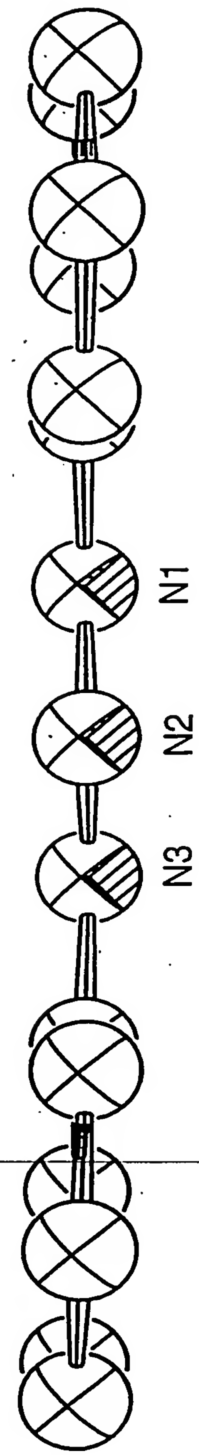


Figure 20

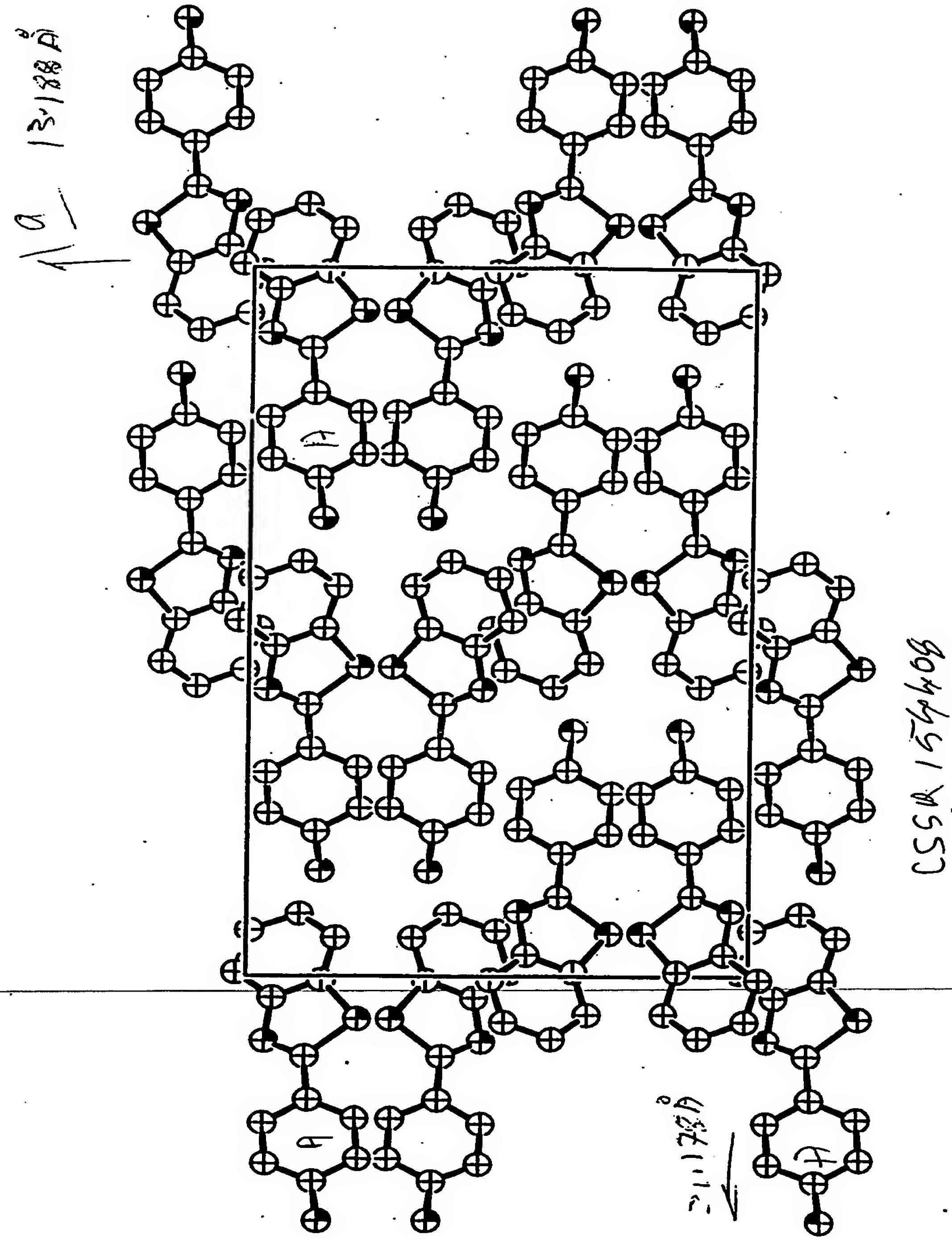


Figure 21

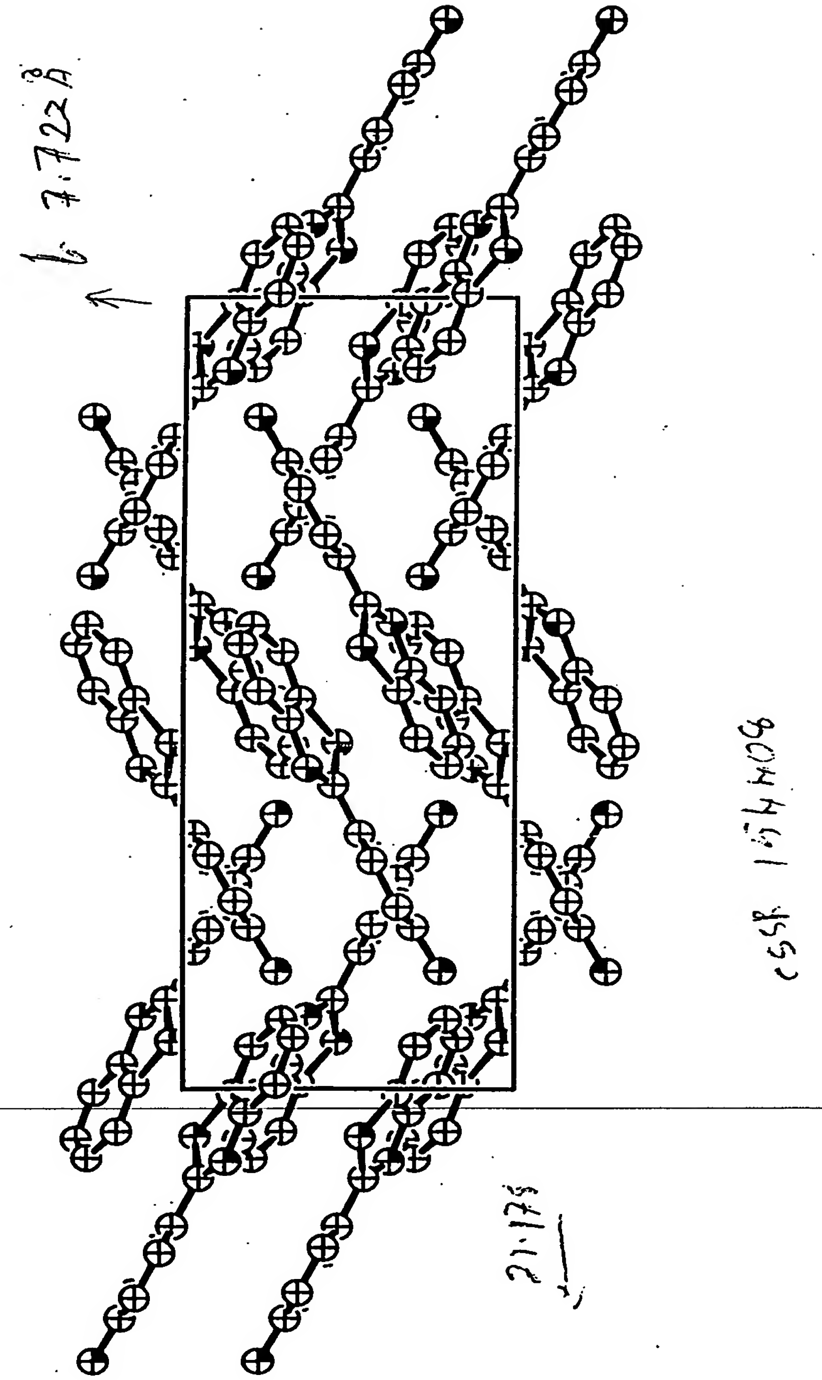


Figure 22

	1 PRIM	2 ANAL	3 THIAZY	4 BENZTHIA	5 MBCC	6 MBNN
Minimum	14.7830	15.0500	15.7270	8.7700	7.0849	9.9600
Maximum	15.1120	15.2610	16.1380	8.9550	7.4443	9.9600
Mean	14.9475	15.1680	15.9273	8.8625	7.3031	9.9600

Figure 23

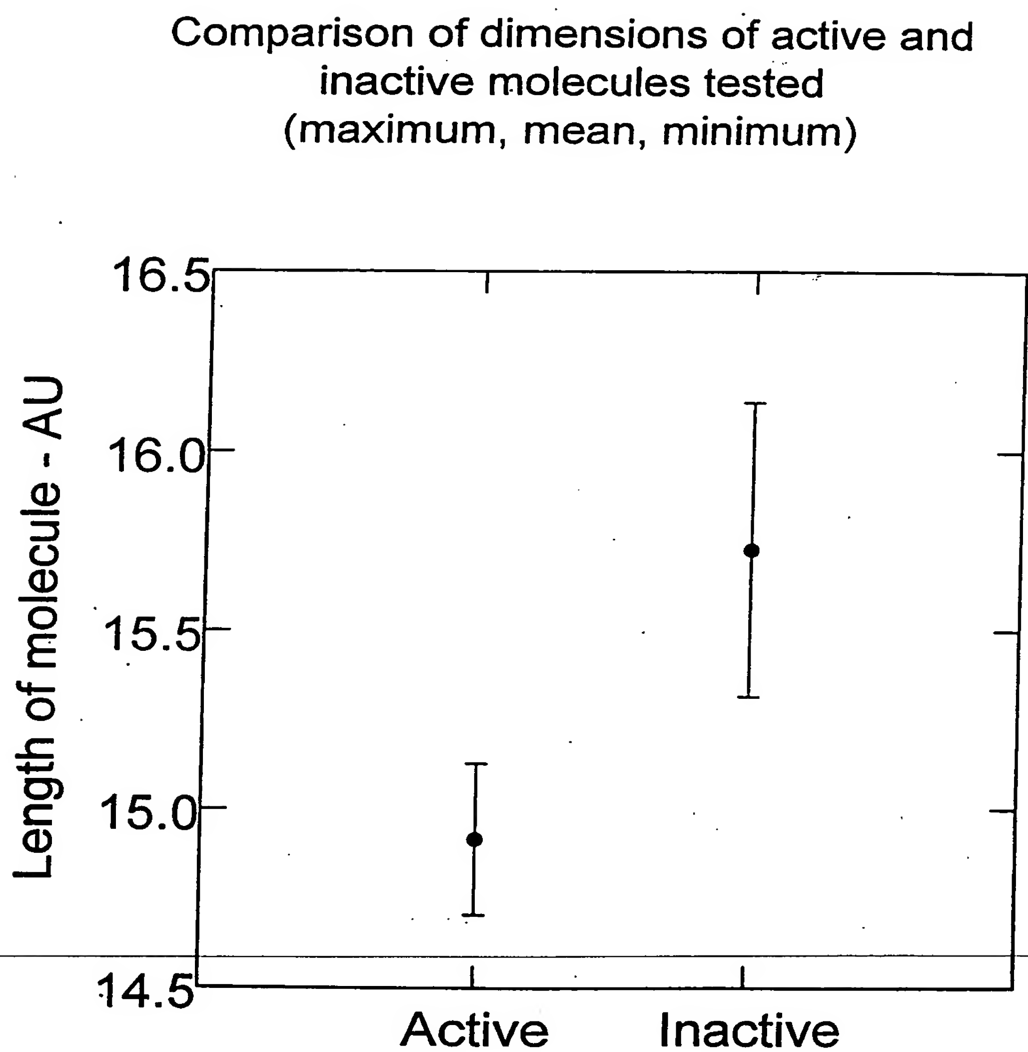
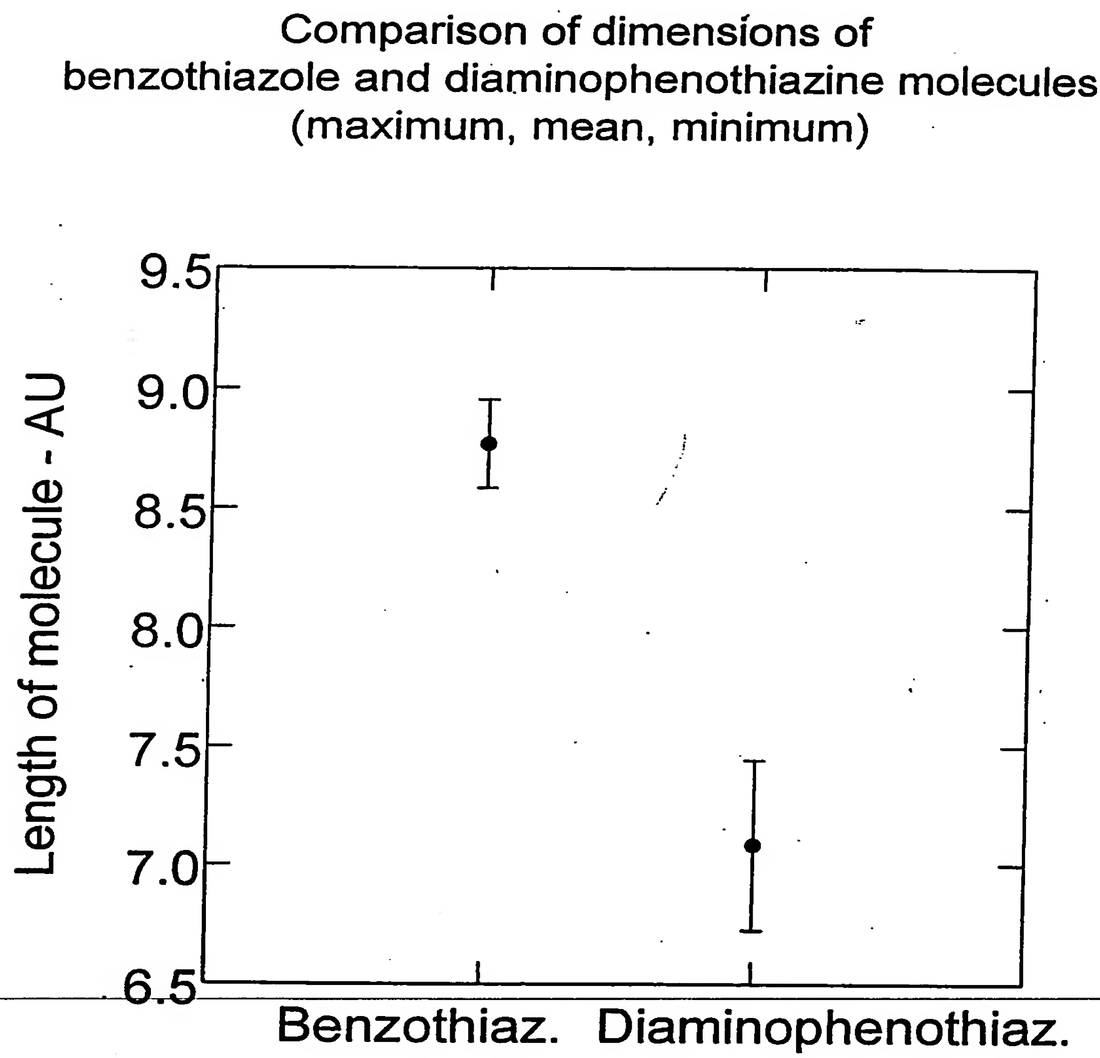

Comparison of dimensions of active and inactive molecules tested (maximum, mean, minimum)
16.5
16.0
15.5
15.0
14.5
Length of molecule - AU
Active
Inactive

Figure 24



Comparison of dimensions of
benzothiazole and diaminophenothiazine molecules
(maximum, mean, minimum)
Length of molecule - AU
9.5
9.0
8.5
8.0
7.5
7.0
6.5
Benzothiaz.
Diaminophenothiaz.

Figure 25

PROBABILITY OF 423-T'S BY REGION AND STAGE

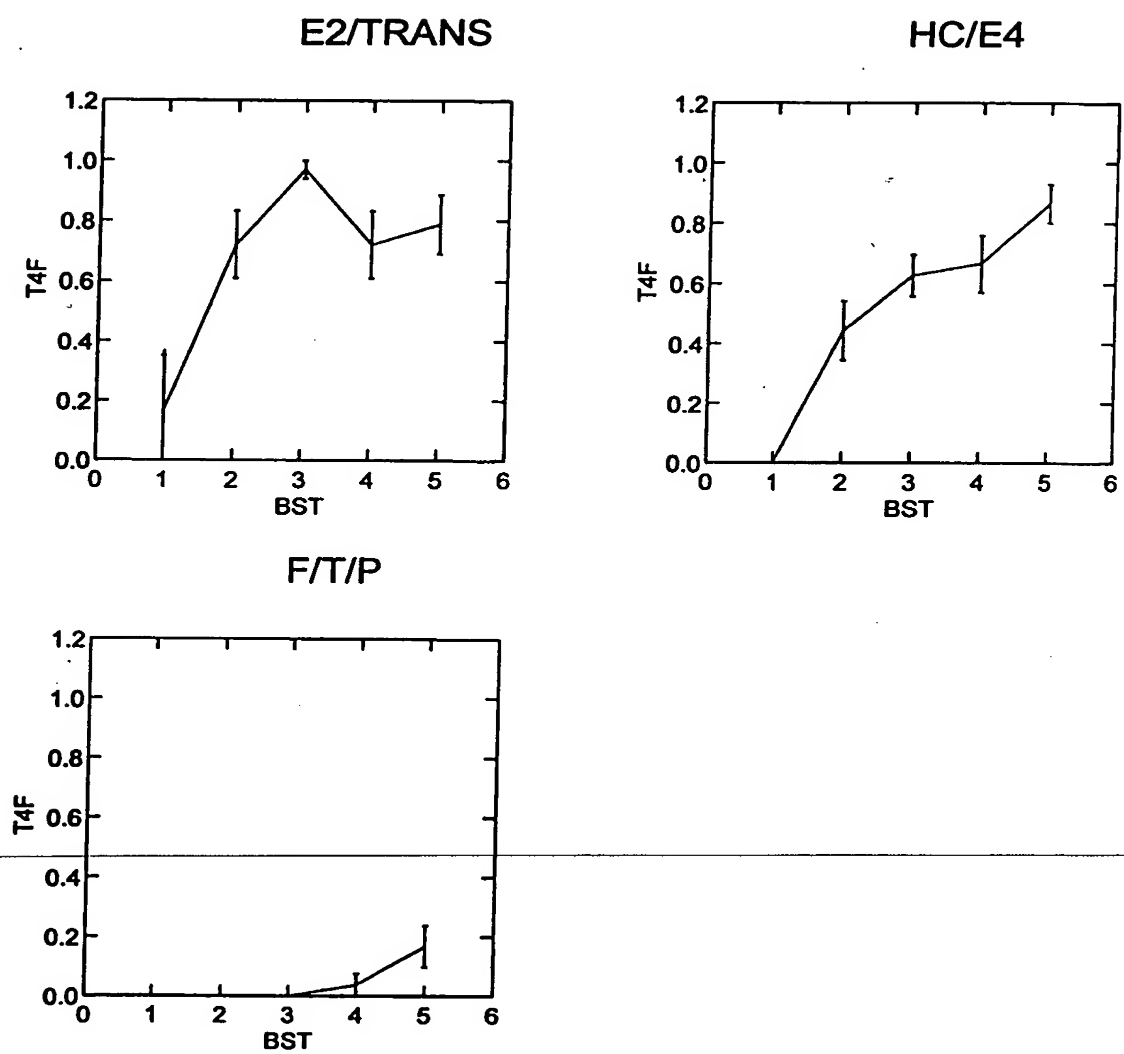


Figure 26

ESTIMATED PHF-TAU CONTENT IN ECT'S BY REGION AND STAGE

REGION	BST	PHF-TAU (μg/g)
E2/TRANS	1	0.62
	2	10.49
	3	23.34
	4	52.46
	5	69.12
HC/E4	1	0
	2	0.83
	3	2.23
	4	5.36
	5	14.36
F/T/P	1	0
	2	0
	3	0
	4	0.08
	5	1.32

Figure 27

PROBABILITY AT8-T'S BY REGION AND STAGE

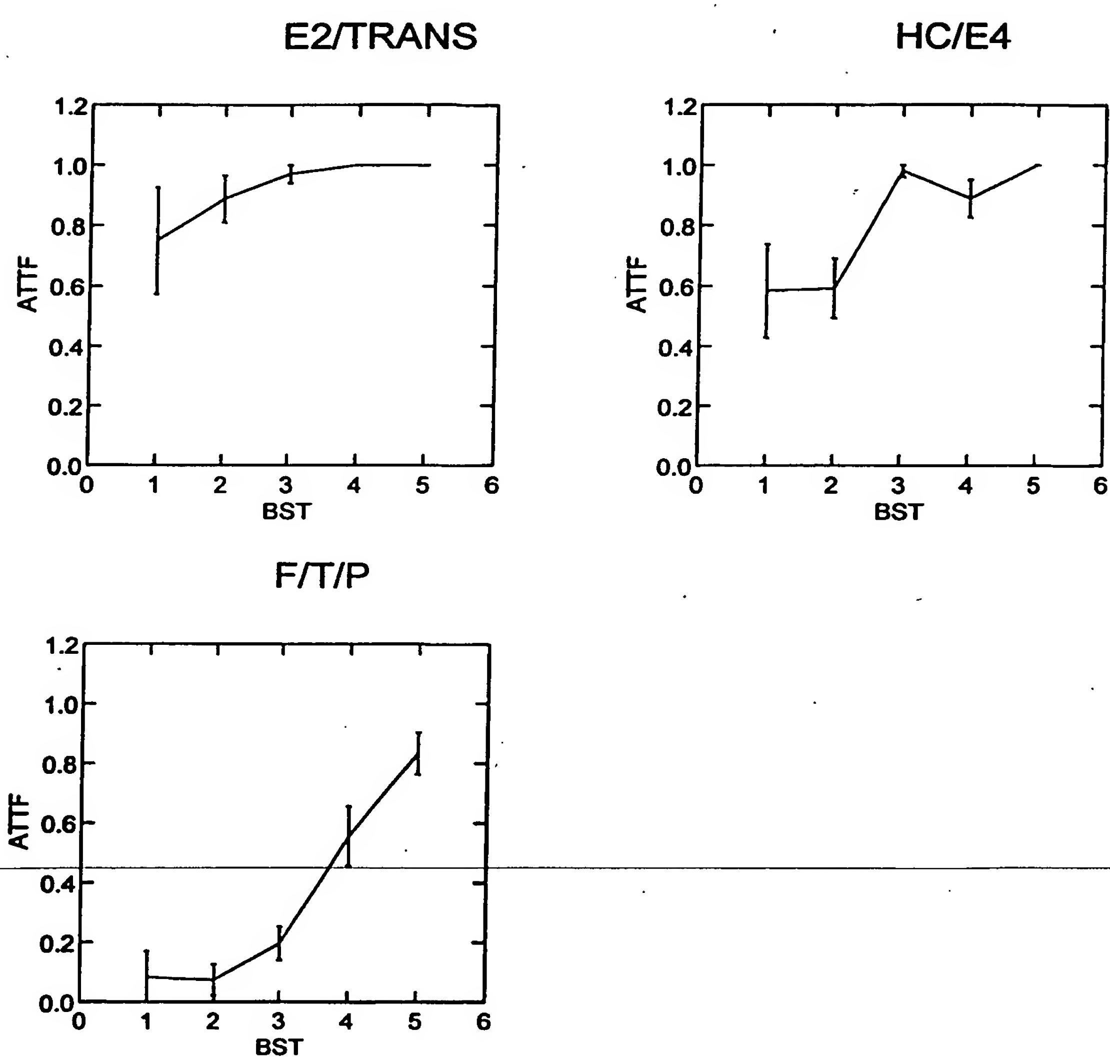


Figure 28

PROBABILITY OF 423-T'S BY REGION AND STAGE
MMSE > 21

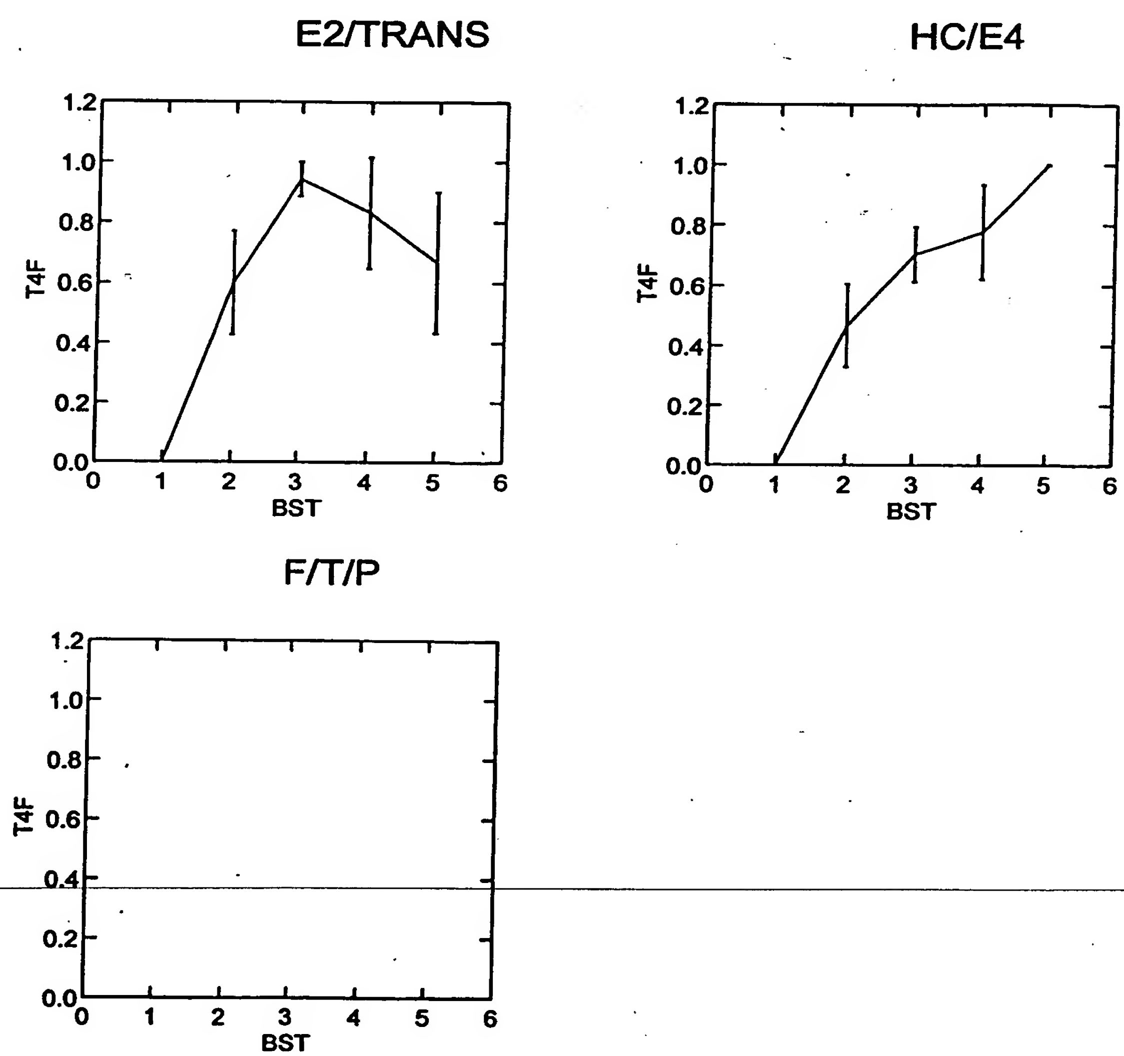


Figure 29

PROBABILITY OF AT8-T'S BY REGION AND STAGE
MMSE > 21

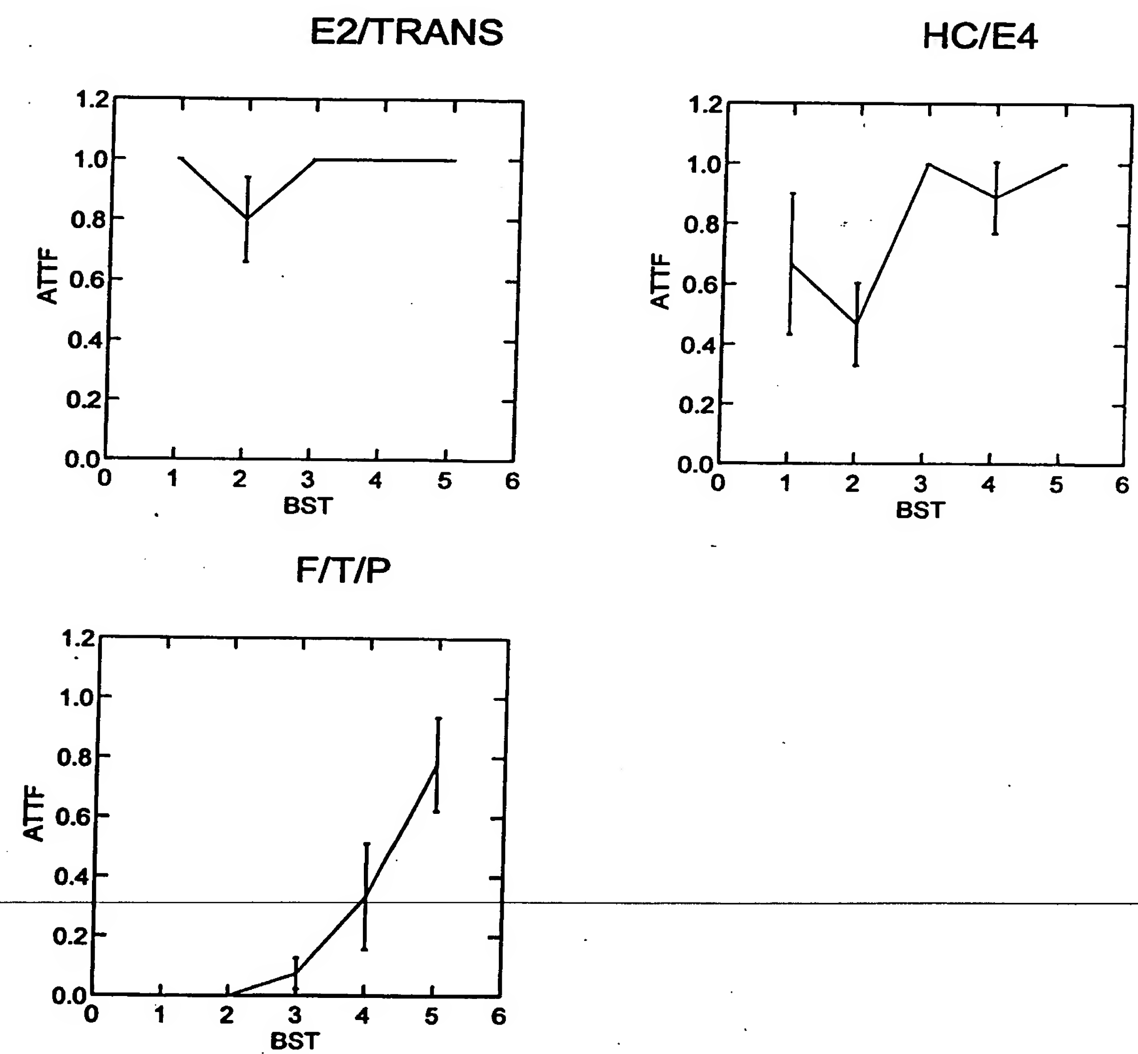


Figure 30

DENSITY OF 423-T'S BY REGION AND STAGE

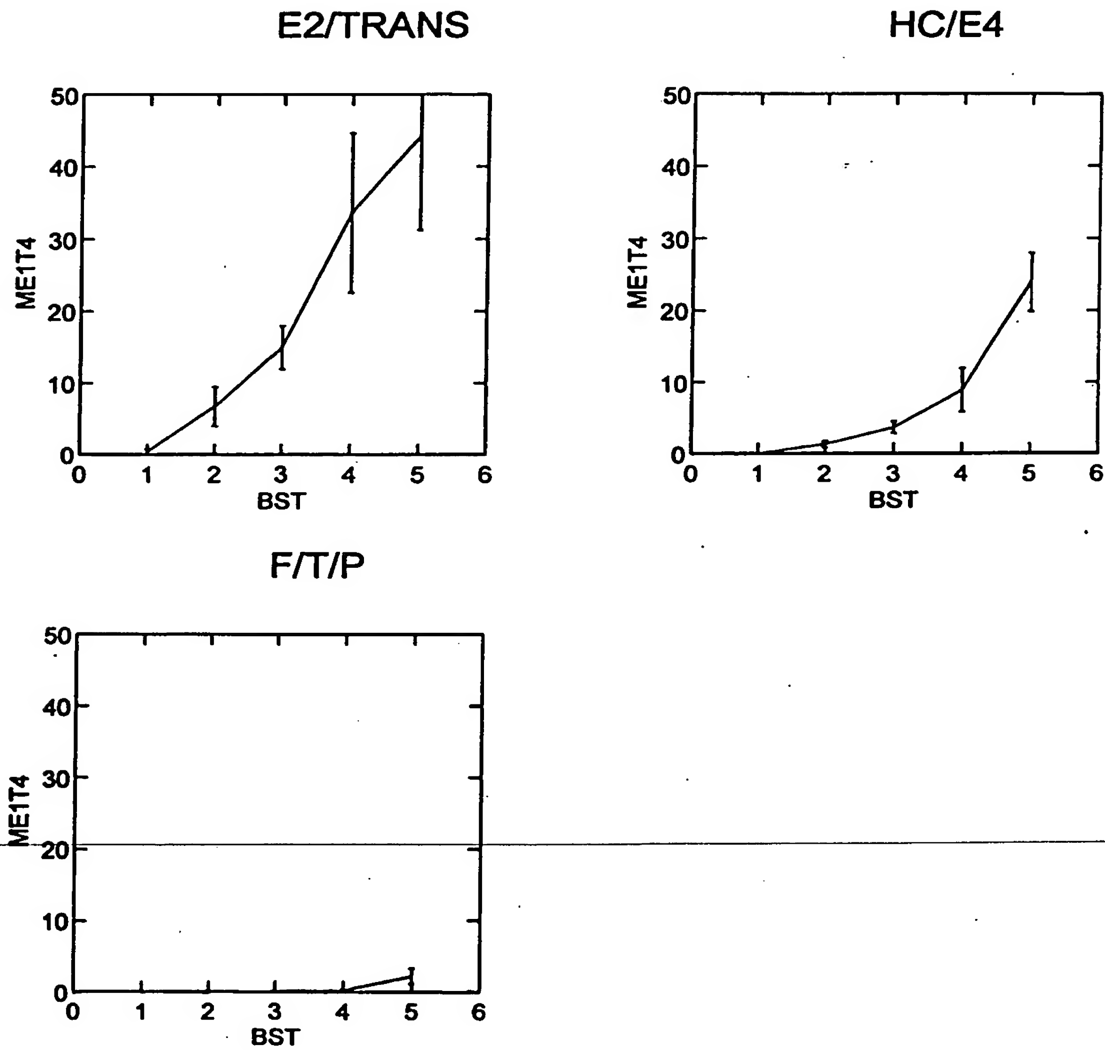


Figure 31

DENSITY OF AT8-T'S BY REGION AND STAGE

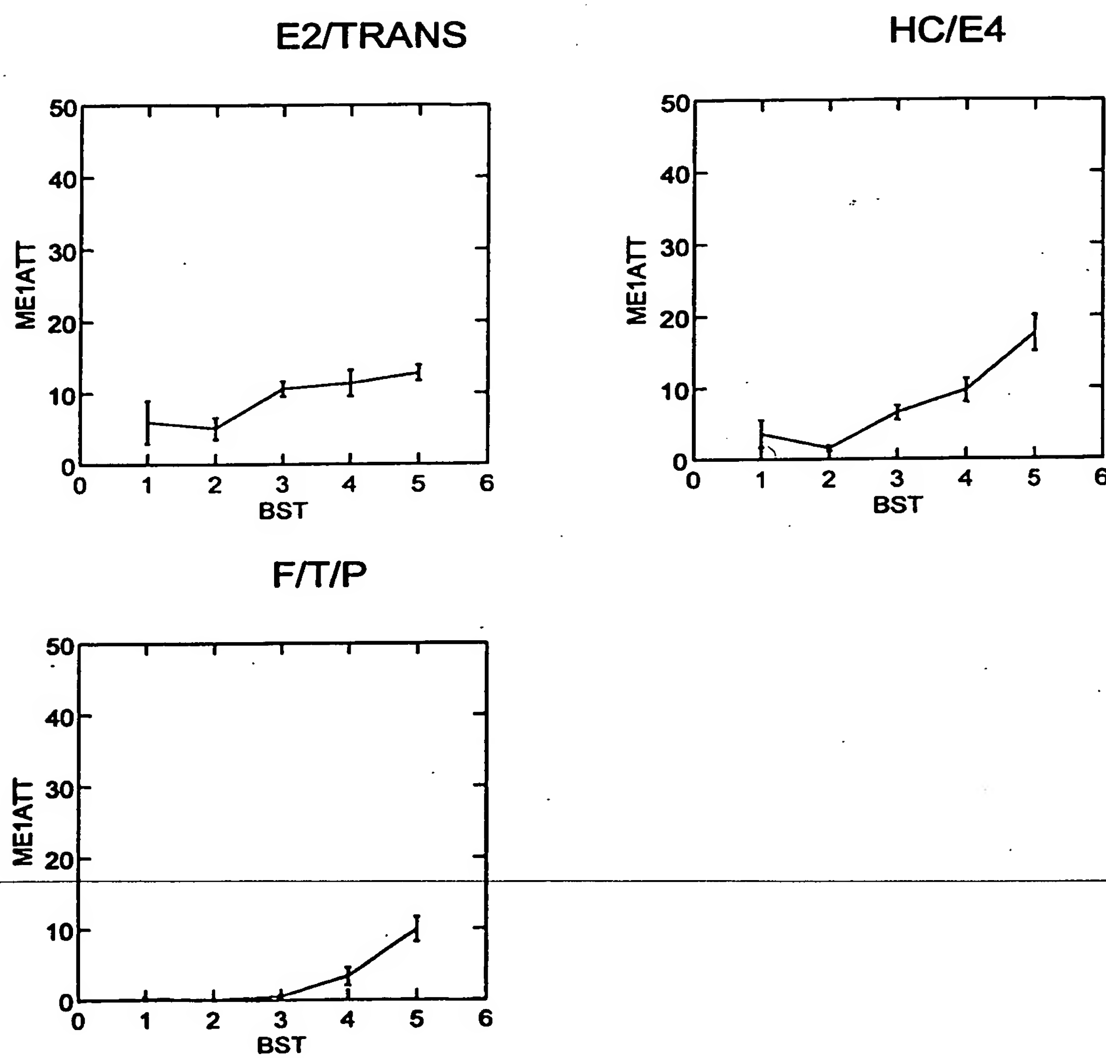
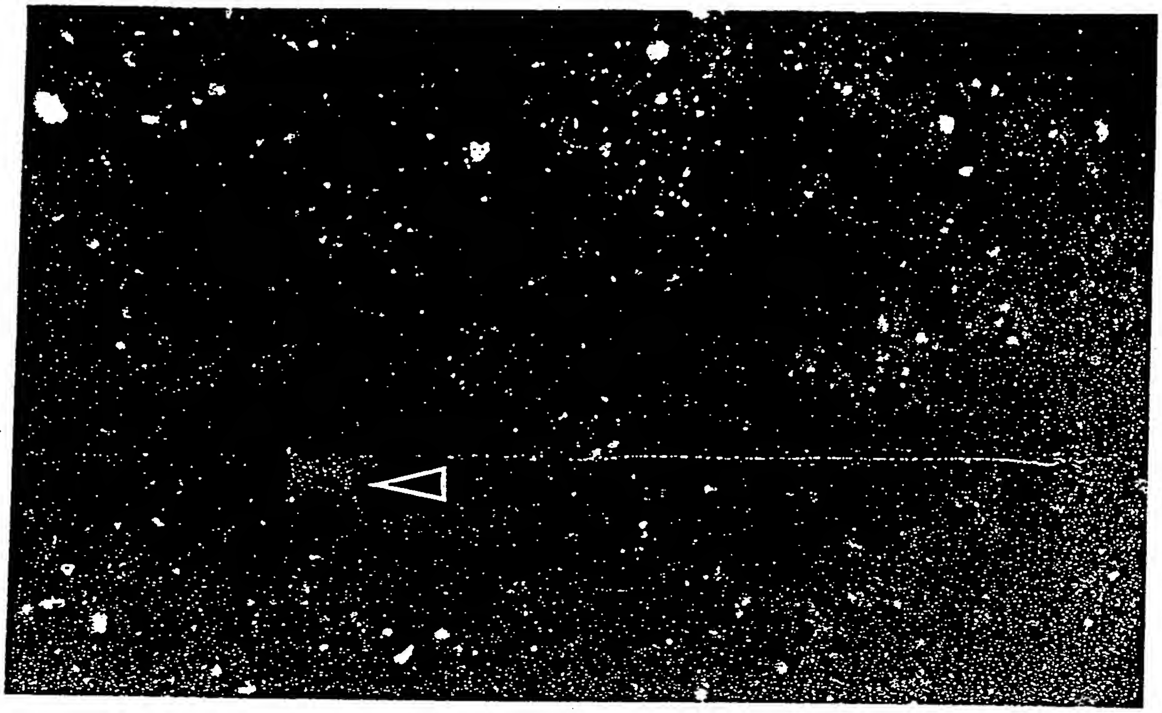


Figure 32



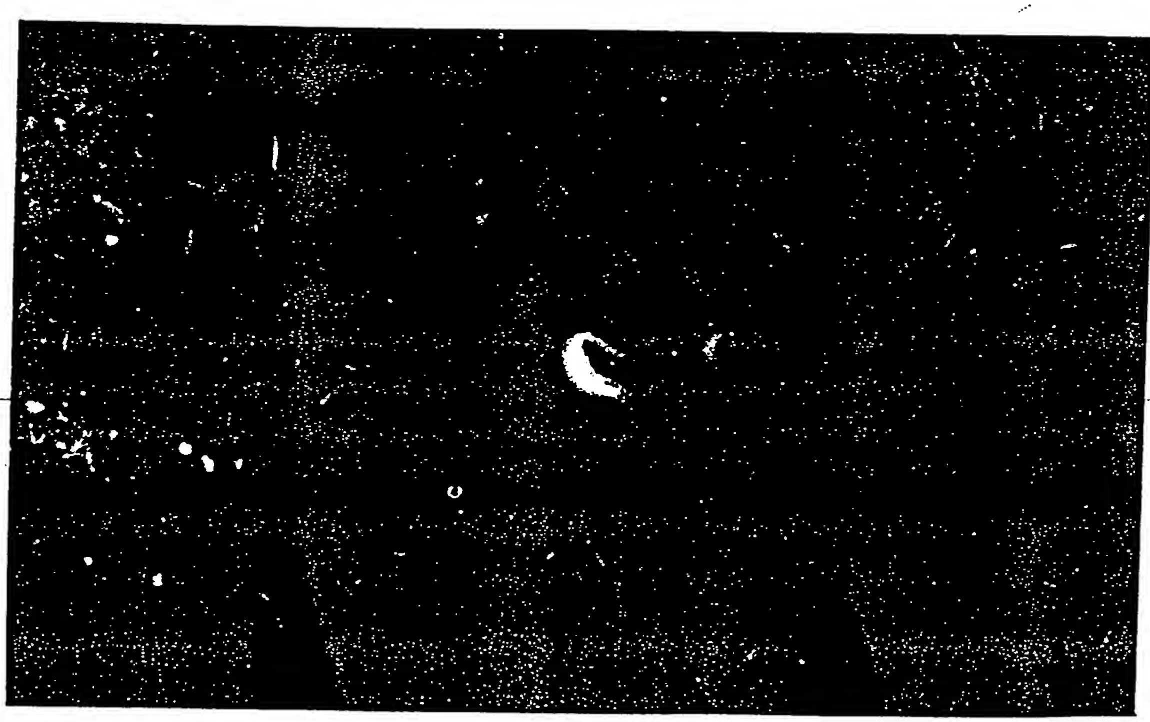


Figure 33